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Elimination of Heterologous Proteins from Blood of Normal, X-Radiated and Tolerant Rabbits.*† (23994)

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Disappearance of serum protein antigens from blood of rabbits proceeds through the following phases: 1) *equilibration*—a rapid initial decrease during first 2 or 3 days as a result of equilibration between intra- and extra-vascular fluid spaces, 2) *exponential elimination*—relatively slow decline which appears to be the result of catabolism of foreign protein in absence of detectable antibody, and 3) *immune elimination*—a rapid decrease, which is the result of combination of antigen with antibody and subsequent catabolism of the complex. Rate of exponential elimination of antigen usually has not been considered to be affected by an immune mechanism(1). However, Cinader and co-workers reported that rate of elimination of human serum albumin (HSA), in normal rabbits, during the exponential phase, was more rapid than the rate in HSA tolerant rabbits, the latter being comparable to the rate of elimination of homologous albumin(2). This would suggest that the exponential elimination of HSA, in the normal rabbit, may be, in part, the result of an immune mechanism which does not necessitate circulating antibody. Such a mechanism would presumably not be operative in the immunologically tolerant animal. To test this possibility, the behavior of serum antigens, in normal, x-radiated and immunological unresponsive rabbits was determined.

Materials and methods. Bovine gamma globulin (BGG), Armour and Co., Lot No. C-904, bovine serum albumin (BSA), Armour and Co., Lot No. P67908, and human serum albumin (HSA), Cutter Laboratories, were used as antigens. These proteins were trace-

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labelled with I^{131} (I*) according to the method of Talmage *et al.*(3). All rabbits were albino males from a single herd. Three groups of immunologically unresponsive rabbits were prepared by several injections, during the first 5 days after birth, with total of 500 mg of either BGG, BSA or HSA(2,4,5). These rabbits were kept without further treatment until they were 3-4 months of age, and weighed 2.0-2.5 kg, at which time they were injected with 10-15 mg of an I* labelled preparation of the same protein. X-radiated§ and/or normal adult rabbits, which were the same age and weight as the above rabbits, were injected with 10 mg of either I*BGG or I*HSA. All rabbits were bled periodically and aliquots of sera, or the trichloroacetic acid precipitates thereof, were counted in well-type gamma counters and the counts converted to % of injected I* protein remaining in the total blood volume. Sufficient counts were obtained to insure a maximum counter error of less than 5%. Half-lives of rates of disappearance of I* protein from blood were obtained by graphic analyses.‖ These analyses were based on exponential elimination of I* proteins which followed equilibration between intra- and extra-vascular fluid spaces.

Results. Eight out of 13 rabbits, injected at birth with BGG, did not show an immune elimination of I*BGG, when I*BGG was injected 3-4 months later, whereas no rabbits injected at birth with HSA or BSA showed an immune elimination of antigen when injected later with the respective I* antigen. All rabbits which did not give an immune elimination of the antigen remained unresponsive when again injected with antigen 1-6 months later. The half-lives of BGG, HSA and BSA in these tolerant animals during the exponential phase were 2.3, 4.0 and 4.3 days respectively. These half-lives were similar to those found for BGG, HSA and BSA in x-radiated and/or normal rabbits (Table I).

TABLE I. Half-Lives of Heterologous Serum Proteins in Normal, X-Radiated and Tolerant Rabbits.

Rabbits	Antigen	Interval of measurement in days after inj.	Avg half-life, days
6 Normal	BGG	2– 5	2.1 ± .2*
6 X-radiated		3– 9	2.2 ± .2
8 BGG-tolerant		3–16	2.3 ± .2
8 Normal	HSA	3– 7	4.1 ± .4
8 X-radiated		3– 9	3.9 ± .3
3 HSA-tolerant		3–17	4.0 ± .4
5 Normal	BSA	3– 6	4.3 ± .3
6 BSA-tolerant		3–20	4.3 ± .2

* Stand. dev.

Discussion. Since rabbits, in which the immune mechanisms have been abolished or circumvented by either x-radiation or induction of tolerance, eliminated BGG, HSA and BSA during the exponential phase at the same rate as do normal rabbits, it would appear that immune factors play no role in elimination of these proteins during this period. This conclusion is in agreement with earlier observations, that half-lives of the exponential elimination of heterologous serum proteins have no relationship to degree of their antigenicity (6,7). However, that these heterologous proteins are recognized as foreign on an apparently non-immunologic basis, is evident from the fact that their half-lives during the exponential phase, in both normal and treated (x-radiated and tolerant) rabbits, were shorter than the half-lives of either homologous albumin or globulin. This can be most readily seen with BGG, which has a half-life of 2.2-2.3 days in either normal, tolerant or x-radiated rabbits, whereas, the half-life of the homologous globulin, in the normal rabbit, is 5.0 days(8). Also, the half-lives of HSA and BSA, in normal and treated rabbits were 3.9-4.3 days, whereas, the half-lives of homologous albumin, in normal rabbits, is 5.7 days(9).

In contradiction to the present observations, Cinader and co-workers(2) reported a significantly longer half-life for exponential elimination of HSA in tolerant than in normal rabbits. Their half-life of HSA in tolerant rabbits was similar to the half-life of homologous albumin. The discrepancy between the present observations and those of Cinader and coworkers cannot be explained. However, the validity of the present data is supported by the fact that similar results were observed with 2 other heterologous serum proteins.

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§ To prevent an immune response to BGG and HSA, some normal adult rabbits were given 400 r wholebody x-radiation 48 hours prior to injection. X-radiation was done at 200 kvp and 15 ma with a filter of 1 mm Al and 0.25 mm Cu delivering at the target distance.

‖ Rate of elimination of BSA from 4 BSA-tolerant rabbits was also calculated from data obtained by duplicate immunochemical determinations. These values agreed, within errors of technic, with those calculated from I* activity.

Summary. Rates of elimination of heterologous serum proteins during the exponential phase are similar in normal, x-radiated and immunologically tolerant rabbits and do not appear to be immunologically determined.

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Antibody Formation in X-Irradiated Rats Protected with Rat or Rabbit Hematopoietic Cells. (23995)

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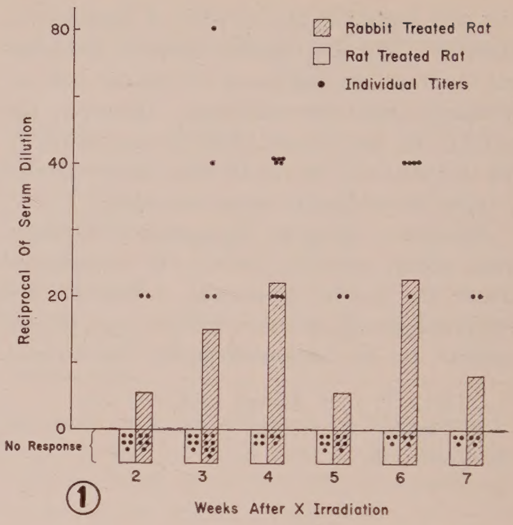
It is now well-established that injection of hematopoietic cells into lethally-irradiated rodents, even of widely divergent genera, results in colonization of the host by the injected cells. Congdon(1) has recently reviewed the status of such colonization experiments. Furthermore, it is apparent that the colonizing cells, even though living in the milieu of their foreign host, maintain their original physiologic properties(2,3,4). Indeed, one of the early proofs for continued existence of such introduced cells was the demonstration(2,3,5) that following injection of rat bone marrow into mice, there is progressive appearance of cells (presumably of rat origin) that stain positively for alkaline phosphatase. Mouse cells do not exhibit this positive reaction. Gengozian and Makinodan(6,7) have tested recovery of antibody-forming ability in lethally-irradiated mice supported with mouse cells and also with rat cells. One can only speculate, however, about whether the antibody-forming capacity in such recovering animals is that of the injected cells or that of the animal's own cells whose recovery is being hastened by supportive treatment.

The present experiments were performed to find out which cells were forming the antibody, using a new technic. Earlier work had shown(8) that embryo liver, which contains predominantly mesenchymal cells, is capable of protecting lethally-irradiated rodents. It is a well-established fact that while rats do not form precipitating antibodies to soluble antigens, rabbits will respond very actively to injections of such antigen(9,10). It is thought that by "protecting" lethally-irradiated rats with rabbit mesenchymal cells and then stimulating them with a soluble antigen one might obtain information on whether injected rabbit cells rather than cells of host rat took part in formation of antibody.

Materials and methods. Male Sprague-Dawley albino rats, weighing 350 to 400 g, were given 750 r total-body X irradiation while confined in a circular aluminum chamber fashioned to hold 12 rats. Delivery source was a G.E. Maxitron-250, operated at 250 KVP and 30 m.a., with 0.5 mm Cu plus 1.0 Al filtration. The half-value layer was

1.43 mm of Cu. Target to center of rat distance was 82 cm, and the delivery rate averaged 28 r/min measured in air at the position to be occupied by the center of the rat's body. Within 4 hrs after irradiation, these rats were injected *via* tail vein, with 1 ml of cell suspension of 28- to 30-day-old rabbit embryo liver, obtained from Swift's snuffle-free rabbits. Livers were removed from freshly-killed rabbit embryos and minced in Locke's solution with sterile scissors. Cells were separated by repeated passage through a 10-ml syringe. The suspension was filtered through gauze to remove coarse clumps. Fatty material, which rose to the surface upon standing, was also removed. The remaining cells were counted with a haemocytometer, and the cell count adjusted to 100 million cells/ml. Two to 7 weeks after liver cell transfer, groups of these rats were injected intravenously with 100 mg of BSA (Bovine Albumin Powder, Fraction V from bovine plasma)* dissolved in 1 ml of *Salmonella typhi* vaccine, prepared by the method of Cannon *et al.*(11). The purpose of the double antigenic stimulus was, on one hand, to obtain information on recovery of antibody-forming capacity to typhoid antigen, to which the rat is normally responsive, and on the other, to search for any response to BSA which normally does not occur in the rat. Rats were bled 7 days after antigenic stimulation, and sera from each group frozen until all samples had been obtained for a simultaneous assay. The method described by Cannon *et al.*(11) was used to carry on agglutination titrations. Since antibody response to BSA might be slight, especially since antigens had been administered intravenously, the anti-BSA titer of sera was determined by the collodion particle method as described by Cannon and Marshall(12), rather than by quantitative precipitation technics, because of greater sensitivity of the former method. Similar groups of irradiated rats were given the same dose of total body irradiation and injected according to the same schedule with 100 million 18- to 20-day-old embryo rat liver cells for control purposes. Representative groups were stressed with BSA plus typhoid antigen at weekly intervals as described above. These controls, of course, served as a check on recovery of capacity to form anti-



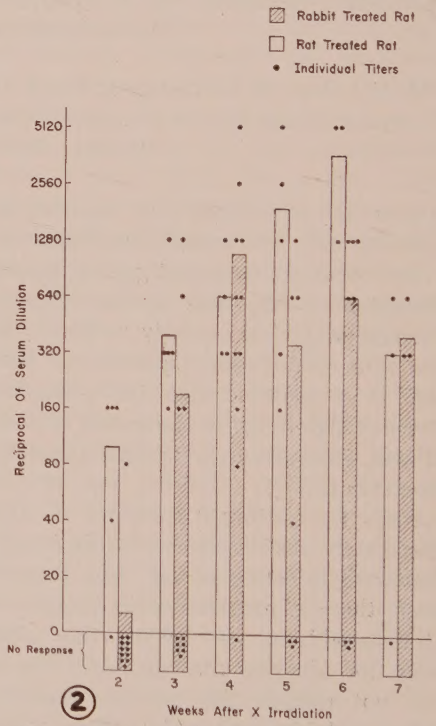


FIG. 1. Anti-BSA serum titers of X-irradiated rats receiving rat embryo liver or rabbit embryo liver cells.

FIG. 2. Anti-typhoid serum titers of X-irradiated rats receiving rat embryo liver or rabbit embryo liver cells.

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* Armour Laboratories, Kankakee, Ill.

body following irradiation.

Results. Average titers to BSA are shown in Fig. 1. Rats irradiated and given rat cells at no time responded to the stimulus of BSA in forming precipitating antibody, nor have normal rats in this laboratory ever done so. Rats which had been given rabbit cells, however, showed a gradual increase in titer, starting as early as first assay at 2 weeks and increasing 4-fold by 4 weeks.

Agglutinin titers to *Salmonella typhi* vaccine on the same rats (Fig. 2) showed that ability to respond to this antigen was fairly well recovered by 2 weeks in rats given rat cells, but in rats injected with rabbit cells, response to the vaccine did not appear to any appreciable degree until 3 weeks. The antibody titer of individual animals is indicated in the 2 Figures by points on each bar representing mean titer of the group. It is apparent that there is some variability in magnitude of titers and that among the rabbit-treated rats there are animals showing no response to either BSA or *Salmonella typhi.* Odell *et al.*(13) have shown that success of recolonization process is not consistent in a group of animals given heterologous cells. Variability in titers and occurrence of negative titers in several rats, apparent in the present work, may reflect variation in recolonization. The fact that the peak of antibody titer occurs at different times after administration of antigen, may also help to explain the variability observed in magnitude of antibody response. Blood was drawn only on 7th day after antigen injection rather than on several days during which peak titer is known to occur in these animals.

Discussion. Irradiated rats injected with rabbit-embryo liver cells formed precipitating antibody in response to BSA. No such response has ever been observed in this laboratory in normal rats or in irradiated rats protected with rat-embryo liver cells. The rabbit responds strongly to stimulation with BSA.

Appearance in these rats of an immune response pattern completely unlike their own and similar to that of donor animal could be due either to presence of rabbit cells capable of antibody formation, or to the fact that rabbit cells administered to save the rat's life following irradiation somehow endowed the immune mechanism of the rat with capabilities typical of the rabbit. Considerable evidence has accumulated in recent years(1) to support the first possibility, but we know of no convincing evidence that cells injected into an irradiated animal have ever resulted in transduction of their traits to cells of the host's body.

In recent independent publications by Weyzen and Vos(14), Grabar *et al.*(15), and Trentin(16) there is speculation on the possibility of colonized cells responding to antigenic stimulation from host's own body proteins to form antibody against the host, thus leading to its death. The results reported above suggest that cells of a colonized heterologous graft are capable of synthesizing a type of antibody that the host's cells cannot manufacture. Thus colonized cells of either isologous or heterologous origin may be considered as surviving and being active in the host's body for a long period of time.

Gengozian and Makinodan(6,7) have shown that agglutinin production in response to antigenic stimulation of sheep RBC returns more slowly when irradiated mice are protected with rat bone marrow than when they are given injections of bone marrow of their own strain. The response observed in rats given typhoid antigen is in keeping with their findings. Agglutinin production in rats protected with rat cells was evident as early as 2 weeks after irradiation and continued to increase markedly at 3rd and 4th weeks, with only 2 rats giving a negative response. On the other hand, rats protected with rabbit cells did not respond to typhoid vaccination at 2 weeks; only 4 out of 11 animals showed an appreciable response by the 3rd week; and not until 1 month was almost entire restoration of antibody response observed. Animals showing no measurable titer were also very common. Presumably, fewer heterologous cells capable of antibody formation are successful in colonizing initially than is true of isologous cells. On the other hand while the initial "take" may be exactly the same in both cases, the subsequent growth rate may be slower in the case of heterologous cells. It is interesting that though this difference is true for recovery of antibody-forming ability, Jacobson(17) has shown that there is essentially no difference if the recovery pattern of total leucocytes in irradiated mice given mouse marrow *vs.* rat marrow.

Heretofore, colonization studies have been most successful within the Rodentia, using mice, rats, or guinea pigs as donors and recipients(1). Jacobson(18,19), however, recently reported that mouse cells successfully enhanced survival in rabbits given 900 r, although the actual presence of these cells in bodies of surviving rabbits has not been demonstrated. Similarly rabbit cells have not been demonstrated in the rat's tissues in our experiments. In this respect, it may be of interest to mention the following preliminary experiment. The only available rabbit that had received 315 million newborn rat-liver cells following 900 r 1 yr previously was given 2 injections of BSA spaced several months apart. Two control rabbits which at that time had been given rabbit-embryo liver cells were treated similarly. These animals were bled 1 week after the 2nd injection.

Whereas the rabbit-treated rabbits responded with a normal precipitin formation, the rat-treated animals gave no response that could be measured even by employing very sensitive technics. Experimental groups are now being studied to expand the observations on this latter approach. Such experiments may aid in establishing just how soon ability to form precipitins is lost, whether it is ever regained by the rabbit following irradiation. The evidence presented above seems to indicate that, under the conditions described, embryonic (mesenchymal) cells other than the rat's own, can survive in the rat body and, after appropriate stimulus, can actively engage in antibody formation for a long period of time after their introduction.

Summary. Rats given lethal doses of total-body X-irradiation were treated with liver cells obtained from rat and rabbit embryos. Two to 7 weeks after this treatment, groups of animals were stimulated with a mixture of Bovine Serum Albumin and *Salmonella typhi* vaccine intravenously. Rats were bled 1 week after antigen injections and titers were performed for detection of antityphoid and anti-BSA antibody. Rabbit-treated rats showed a positive titer to BSA and a delay in recovery of antibody-forming capacity to both antigens administered. Although rat-treated rats recovered their antibody-forming capacity earlier, they did not show any response to BSA. Possible implications of these phenomena are discussed.

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Fluorescent Antibody Staining of Street and Fixed Rabies Virus Antigens (23996)

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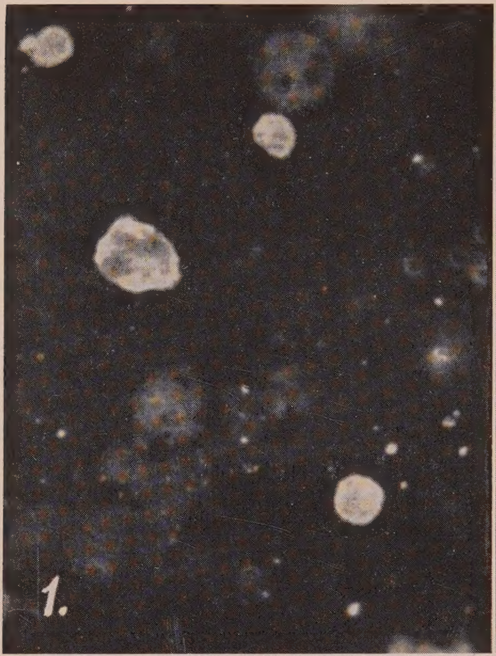
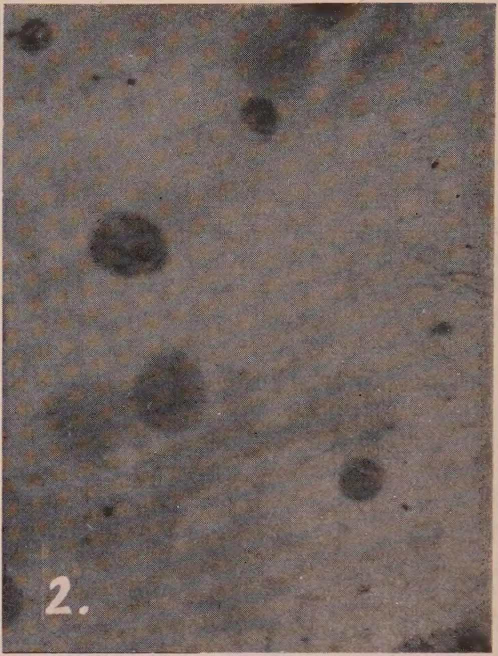
The diagnosis of rabies in animals is usually based upon the demonstration of Negri bodies in brain smears or sections. However, these inclusion bodies cannot be demonstrated in approximately 10% of naturally infected animals and under certain conditions this percentage may rise to 26%(1). These Negri negative animals may be confirmed as infected with rabies by virus isolation procedures but such technics are too time-consuming to be used as basis for decision regarding vaccination. Because of dangers inherent in the use of the standard anti-rabies vaccines, it is desirable to administer them only to persons who have experienced a real exposure to the virus. Thus there is need for a diagnostic technic which will combine the speed of histological technics with the accuracy of biological examination.

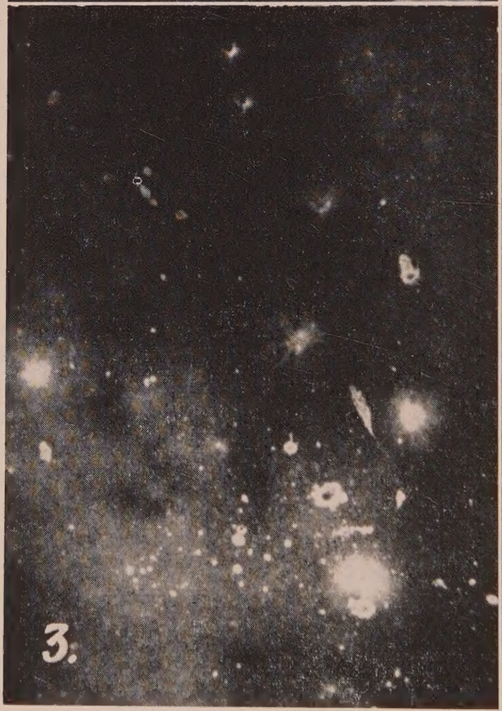
Materials and methods. Strains of rabies virus we used included fixed virus strain CVS 11 obtained from National Institutes of Health, Division of Biologics Standards, and several strains of street virus isolated from salivary glands of dogs and foxes in Alabama. These strains were prepared as 20% infected mouse brain suspensions in a diluent consisting of 10% yolk from 6-7-day embryonated eggs in .05 M sodium phosphate buffered water, pH 7.6-7.8. All suspensions were lightly centrifuged to remove larger particles before storage at –60°C. Mice were inoculated intracerebrally and were sacrificed on showing definite signs of disease; the brains were removed and impression smears made from cross-section which included the hippocampi. Various methods of fixing were tested but only one gave consistently satisfactory results. Freshly prepared smears, while still moist, were dropped into Coplin jars containing acetone pre-cooled in dry ice-alcohol bath. The jars were then transferred to a household type deep freezer kept at –15° to –20°C. After 18-24 hours, slides were removed from the acetone and drained and dried without being taken out of freezer. These slides stained satisfactorily with fluorescent antibody even after prolonged storage. Indirect staining(2), complement staining(3) and direct staining(4) have all been used in this work. The conjugates used for indirect stain were rabbit-antihuman globulins and rabbit antiguinea pig globulins conjugated with use of fluorescein impregnated filter discs as described elsewhere(3). Rabies antiserums were derived from humans who had undergone an experimental course of immunization with duck embryo grown rabies vaccine. Following fixing and drying in the cold, the impression smears were treated with antiserum diluted 1:10 or with a mixture of equal parts antiserum diluted 1:5 and guinea pig complement diluted 1:10. These slides were incubated 1/2 hour at 37°C in a wet chamber, washed 10 minutes in .01 molar phosphate buffered saline pH 7.4-7.6, exposed to the appropriate conjugate at 37°C for ½ hour, washed and mounted in buffered glycerol. For direct stain, a commercial refined and concentrated horse antirabies serum (Lederle) was used. This serum was simply diluted to suitable concentration for labeling without any preliminary treatment, and conjugation was carried out as above. All conjugates were absorbed once with hamster liver powder and again with mouse liver or mouse brain powder (5), to reduce nonspecific staining.

Results. Street virus. Fig. 1 illustrates typical staining obtained in impression smears of brains from mice in late stages of rabies infection. Great variation was seen in size of fluorescent bodies which ranged from size of nuclei of nerve cells or larger, to the barely visible. That these were associated with rabies virus was borne out by the following evidence:

A. Paired (pre-immunization and post-immunization) serums from the same individuals were used to stain duplicate slides. The pre-







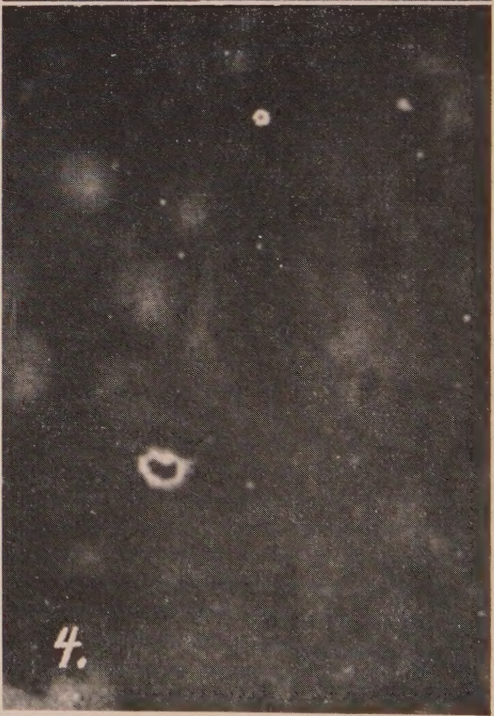


FIG. 1. Impression smear of brain from mouse infected with street rabies virus, stained with fluorescent antibody. × 620.

FIG. 2. Same field as in Fig. 1, stained with Shleifstein's stain. × 620.

FIG. 3 & 4. Impression smear of brain from mouse infected with CVS 11 strain of fixed rabies virus stained with fluorescent antibody. Fig. 3, × 620; Fig. 4, × 530.

immunization serums did not give staining, whereas post-immunization serums resulted in good to excellent staining, depending on individual serum.

B. Since immunization had been carried out with duck embryo grown vaccine, it was considered unlikely that staining was due to production of antibodies against normal mouse proteins, and in fact smears made from normal mouse brains failed to show staining with either pre-immunization or post-immunization serums. The possibility that immune serums contained antibodies against abnormal mouse antigen formed in response to virus-induced encephalitis was investigated. Groups of mice were inoculated intracerebrally with various dilutions of St. Louis encephalitis virus and pseudorabies virus. No staining was obtained in brain impression smears of these mice with any of the rabies immune serums.

C. In indirect staining procedure as usually carried out, the antigen, which is fixed on a slide, is treated with antiserum diluted in saline and then with a fluorescein-conjugate directed against the antiserum globulins. However it was noted that when an antiserum is suitably diluted not in saline but in a solution or suspension of antigen immunologically related to that fixed on the slide, subsequent staining will be partially or completely inhibited, depending on the closeness of that immunological relationship(6). The rabies-immune serums were therefore diluted 1:10 in a 20% suspension made from brains of mice in late stages of infection with CVS 11 virus, and in a similar suspension made from brains of normal mice. Impression smears from street rabies-infected mice were then treated with each of the above mixtures. The smears treated with antiserum diluted in normal mouse brain showed good fluorescent staining with very low background whereas those treated with antiserum diluted in infected mouse brain gave no visible staining.

D. The number and distribution of the larger fluorescent bodies corresponded well with the number and distribution of Negri bodies seen in duplicate slides stained with Sellers' stain. Some of the fluorescent bodies showed quite uniform fluorescence, but others showed non-homogeneous staining (Fig. 1). In some cases fluorescent inner granules could be seen, but this was the exception rather than the rule, and it was not found possible to determine whether these corresponded to the inner granules, diagnostic of Negri bodies, seen when stains such as Sellers' are used.

Many attempts were made to remove cover slips from smears stained with fluorescent antibody and to stain the same preparations with one of the conventional Negri body stains. The results were invariably poor. Staining was very weak and though the Negri bodies could be seen, the inner granules were rarely visible. When present they were in the Negri bodies in regions where the smear was thickest, areas not suitable for examination with the fluorescence microscope due to high autofluorescence.

Fig. 2 shows the same field as Fig. 1, following the use of Shleifstein's stain. But whereas all large fluorescent bodies stained with above dyes, only some of the smaller ones did so and these could not be distinguished from acidophilic particles which had not shown fluorescent staining.

Fixed virus. Fig. 3 and 4 show some examples of the staining obtained in impression smears of brains from mice in late stages of infection with the CVS 11 strain of fixed rabies virus. No large round fluorescent bodies were seen as with street virus, but many of the smaller particles were identical in appearance to those seen in street virus infections. In the fixed virus, "ring" forms were relatively frequent in appearance, some quite large (Fig. 4). When cover slips were removed from such preparations and the smears stained with Giemsa or Shleifstein's stain, some of these rings and other particles could be found with difficulty since they stained very weakly, but they could not be distinguished from other objects similar in appearance which had failed to show fluorescent staining.

The same tests for specificity of staining were applied as with street virus, with identical results, *i.e.*, normal mice or mice infected with encephalitic viruses other than rabies failed to show staining; pre-immunization serums did not stain with fixed rabies-infected mouse brain whereas post-immunization serums from the same individuals did so; finally, mixing immune serums with rabies-infected mouse brain suspensions completely inhibited staining of smears but similar use of normal mouse brain suspensions caused no such inhibition.

Discussion. It has long been assumed that Negri bodies contain virus particles and cytochemical studies(7) have presented indirect evidence for this hypothesis. The major part of Negri bodies, however, has been thought to be composed of a matrix derived from cytoplasm of degenerating cells(1). Fluorescent antibody studies, however, indicate that these inclusion bodies contain concentrations of viral antigens which seem to be distributed diffusely throughout the Negri bodies rather than being restricted to the inner granules. The antibodies used in indirect staining of mouse brain smears were produced with virus grown in the duck embryo, thus precluding the likelihood of formation of antibody to mouse tissue. No staining of normal mouse brain was observed, nor did rabies-immune serums produce staining of smears from mice infected with St. Louis encephalitis or pseudorabies virus. Diluting antisera in normal mouse brain suspension reduced background fluorescence to a minimum but had no inhibiting effect on brightness or type of staining, whereas diluting the antiserum in rabid mouse brain suspension caused complete inhibition of such staining.

Small eosinophilic inclusion bodies, which cannot ordinarily be considered as specific because of their similarity to inclusion bodies found in other diseases, are often seen in brains of animals infected with street rabies virus. Fluorescent antibody staining, however, indicates that some of these at least are of rabies virus origin. Actually, staining of particles of all sizes down to the barely visible was seen, and these possibly represented aggregates of various numbers of elementary bodies of the virus. It was not possible to differentiate brain smears of fixed virus-infected mice and street virus-infected mice except that in the latter larger fluorescent bodies appeared, apparently representing the Negri bodies.

Since it was possible to demonstrate specific staining of fixed virus which does not form Negri bodies, as well as of street virus which was not included in Negri bodies, it appears that this type of staining might be useful for rapid diagnosis of rabies in Negri negative animals. Furthermore, the fact that excellent staining was obtained with antisera derived from humans immunized with a rabies vaccine, indicates that this might furnish a useful tool for studying development of immunity in man or animals undergoing rabies vaccination.

Preliminary studies have shown that the methods described may be suitable also for demonstrating the presence of rabies virus in the salivary glands of naturally infected animals. Staining occurred even in glands which had been immersed in glycerine for periods as long as 3 years. Small pieces of glands were rinsed free of glycerine and then used to make impression smears. In some cases triturated tissues were used. The smears were dried and stained as described above. Although large amounts of autofluorescent material were usually present in these smears, the yellow-green fluorescence of the stained antigens could be readily distinguished. The antigens in the glands were similar in appearance to those observed in the brain smears except that the fluorescent bodies tended to be smaller.

Positive glands from foxes, dogs, cows, skunks and one wildcat have been examined so far by this method and a field trial is now in progress in collaboration with the Alabama State Health Department laboratories to determine the reliability of this procedure for determining the presence or absence of virus in the salivary glands of suspected animals.

Many strange and bizarre forms of fluorescent staining material were found in smears of both fixed and street virus in addition to those shown in the photographs. It seems probable that these were due simply to artificial distortion of the antigenic material during the making of impression smears. Staining of frozen sections should make it possible to determine whether these bizarre forms or the ring forms shown in Fig. 3 and 4 exist as such in the animal tissues or whether they are artifacts resulting from the methods used in making and fixing the smears.

Summary. Methods of fixing and staining of rabies-infected mouse brain smears with fluorescent antibody are described. Evidence is presented that Negri bodies are of viral origin. The possible usefulness of this method for diagnosis of rabies in Negri negative animals and for serological determinations following immunization against rabies is indicated.

Preliminary studies indicate that this technic may be useful in the detection of virus in salivary glands.

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Growth of Rabies Virus in Non-Nervous Tissue Culture. (23997)

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Successful attempts to grow rabies virus in tissue culture have been summarized by Sanders and co-workers(1). The essential feature of each report was the use of brain tissue from embryonic or very young mice, rabbits, or chicks. Previously, attempts to propagate rabies virus in non-nervous tissue cultures have been unsuccessful(2,3). Vieuchange and co-workers(4) reported that fixed rabies virus could be recovered from mouse kidney cultures inoculated 20 days previously. However, they did not demonstrate an increase in virus nor present adequate evidence that the virus could be serially propagated in mouse kidney cultures.

Having demonstrated successful propagation of several neurotropic viruses in hamster kidney cultures(5) these tissues were used in an attempt to propagate rabies virus.

Materials and methods. Cell suspensions of hamster kidneys (HK) were prepared as described previously(5). During the earlier phase of this study the nutrient medium consisted of 3-5% calf serum and 0.5% lactalbumin (LA) hydrolysate in Hanks' balanced salt solution (HBSS) plus antibiotics. Later, the nutrient medium used for infected cultures consisted of 20% horse serum, 0.5% LA in HBSS, although the original medium was still used for growth of the cells until the time of inoculation with virus at 5-14 days of age. Tissue cultures were inoculated with 0.2 ml volumes. Mice used for detection and titration of virus were 3 weeks old, of the CFW strain, and were inoculated intracerebrally with volumes of 0.03 ml. The CVS strain of fixed rabies virus was used. The rabies street viruses used were in the form of naturally infected salivary glands, 2 from dogs and one from a cow, all cases occurring in Alabama during the summer of 1957.

Results. Fixed rabies virus. Hamster kidney cell cultures were inoculated with 0.1 ml of a 10^{-3} dilution of fixed rabies-infected mouse brain representing approximately 10,000 mouse LD_{50} units. The nutrient fluid was harvested and replaced with fresh fluid at 2-4-day intervals over a period of 91 days. No cytopathic changes occurred during this period. Twenty-seven such changes of fluid were made during this period and rabies virus was demonstrated in each harvest, the mouse LD_{50} titers varying between $10^{0.6}$ and $10^{5.3}$. In several instances the amount of rabies virus recovered was somewhat greater than the amount originally inoculated, the greatest in-

TABLE I. Virus Yield from Hamster Kidney Tissue Cultures Infected with Fixed Rabies Virus.

HKTC passage No.	Day of harvest	LD_{50} titer in mice ($\log_{10}$)
1	35	3.3
2	10	1.8
3	15	2.5
4	17	3.1
5	14	4.0
6	15	4.5
7	10	3.5
8	11	3.0
9	14	3.8
10	11	2.8
11	10	3.5
12	10	3.3
13	10	3.8
14	10	4.3
15	10	3.5

dicating approximately a 100-fold increase in virus.

Fluid harvested the 6th day, which had a mouse LD_{50} titer of $10^{4.0}$, was inoculated into fresh tubes of kidney cultures. Small amounts of virus could be demonstrated in the fluid from these cultures on the 6th day after inoculation but attempts to accomplish further serial passage in tissue culture from this material failed.

Attempts were then made to adapt the virus to the HK cells by alternate passage between mice and HK cells, harvesting the fluid from the cell cultures on the 6th or 7th day after inoculation. Six complete alternating passages were accomplished without difficulty but efforts to maintain the virus in serial HK passage at each level failed after one or 2 HK passages.

The fluid taken at 35 days from the cell cultures inoculated with the mouse strain virus mentioned above was inoculated into fresh cell cultures. This inoculum contained $10^{3.3}$ mouse LD_{50} units per 0.03 ml. By attempting passage at various intervals after inoculation of these cell cultures it was found possible to accomplish serial passage of fixed rabies virus in HK cells through at least 15 passages without any diminution in titer of the virus (Table I). Titrations of the fluids from inoculated cultures revealed that the maximum virus titer was not usually reached until the 10th to 15th day after inoculation. Serial tissue culture passage was usually unsuccessful when the inoculum titered less than $10^{1.5}$ LD_{50} in mice. No cytopathic changes could be observed in cell cultures infected with fixed rabies virus at any passage level.

Since it appeared that virus was being released continually by the cells, it was reasoned that an increase in the protein content of the medium would prevent some inactivation of the virus at incubator temperatures, thereby permitting a greater accumulation of viable virus. Experimentally it was found that progressively more virus could be recovered from cultures in which the media contained progressively more protein (Table II). When media containing either monkey, rabbit, guinea pig, calf, or horse serum were compared it was found that infected cells in media containing horse serum yielded the most virus (Table III).

TABLE II. Effect of Composition of Nutrient Medium upon Yield of Rabies Virus from Hamster Kidney Cultures. (Harvest at 12 days.)

Medium	Virus yield $\log_{10}$ LD_{50} in mice
HBSS	.5
.5% LA in HBSS	1.1
3% CS, .5% LA in HBSS	1.3
10% CS, *Idem*	2.3
20% CS, ”	2.8

HBSS = Hanks' balanced salt solution. LA = Lactalbumin hydrolysate. CS = Calf serum.

The virus recovered at the 15th passage through hamster kidney cultures was confirmed as rabies virus by neutralization tests in mice.

Street rabies virus. Three preparations of street rabies virus were inoculated into HK tissue cultures. A 10^{-2} dilution of naturally

TABLE III. Effect of Species Origin of Serum Used in Medium upon Yield of Rabies Virus from Hamster Kidney Cultures. (Harvest at 7 days.)

Species serum in medium*	Virus yield $\log_{10}$ LD_{50} in mice
Rabbit	1.5
Guinea pig	1.8
Monkey	2.1
Calf	2.8
Horse	3.2

* All media contained 10% serum, .5% LA in HBSS.

TABLE IV. Virus Yield from Hamster Kidney Tissue Cultures Infected with Street Rabies Virus.

HKTC passage No.	Day of harvest	LD_{50} titer in mice ($\log_{10}$)
1	24	1.5
2	14	4.3
3	13	4.3
4	11	4.0

infected salivary gland was used in each case. No virus could be subsequently recovered from the tissue culture fluids which had been inoculated with 2 of the specimens. One of these consisted of dog salivary glands with a titer of $10^{6.5}$ LD_{50} in mice, the second a suspension of cow salivary glands with a mouse LD_{50} titer of $10^{5.3}$.

The fluids from tissue cultures inoculated with a third suspension, dog salivary glands having a titer of $10^{6.1}$ LD_{50} in mice, yielded virus at each change of fluid made every 3-4 days through 39 days after inoculation. This virus was capable of being serially passed at least 4 times through HK tissue cultures without a drop in infectivity titer for mice (Table IV). Negri bodies could be demonstrated in impression smears from the brains of mice inoculated with tissue culture fluids at each passage level. No cytopathic changes were observed in the tissue cultures.

Summary. Both fixed and street rabies virus strains were propagated serially in hamster kidney tissue cultures but no cytopathic changes were evident in these cultures.

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Detection of Gastro-Esophageal Reflux by Simultaneous Measurement of Intraluminal Pressure and pH. (23998)

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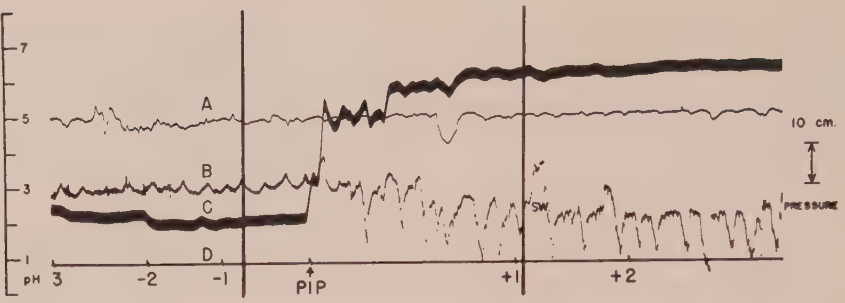
Measurement of pH at various levels within the esophageal lumen provides a direct means for detecting gastro-esophageal acid regurgitation, and simultaneous recording of intraluminal pressure changes permits precise localization of the sensing device at various levels above the diaphragmatic hiatus.

Method. 300 ml of 0.1 N HCl are introduced into the stomach of a fasting subject to assure the presence of pH 1 to 2 for at least 30 minutes. Smaller quantities of acid fail to maintain these pH levels in individuals with rapid gastric emptying. An exploring glass pH electrode on a long flexible lead (Beckman No. 78022V) and small-caliber, water-filled polyvinyl tube are then passed together through the nares into the esophagus. The open end of polyvinyl tube is at the level of bulb of glass electrode, and this relation is maintained by tying tube to lead wire of electrode with fine silk thread. The polyvinyl tube measures 1.6 mm (internal) and 2.5 mm (external) diameter. Both tubes measure 4.8 mm diameter. The polyvinyl tube is attached to pressure transducer (Statham Model P6a ± 2D-300) by a 3-way stopcock; a syringe attached to latter permits flushing of tube with water during the test. Direct observations of pressure changes are made on a mirror galvanometer which receives the output of the pressure transducer. The exploring glass pH electrode is attached to a pH meter (Beckman Zeromatic, Model 9600) with circuit established by having subject immerse his unabraded finger into saturated solution of KCl containing the calomel electrode. This



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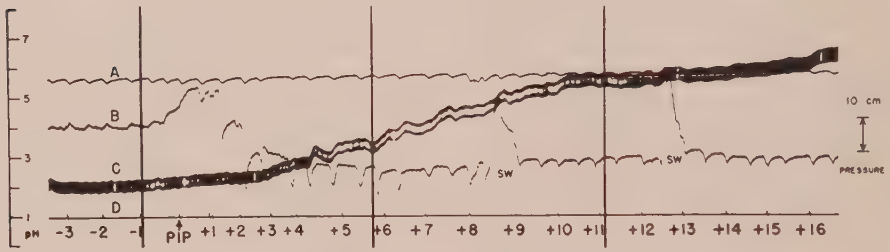

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FIG. 1. Record obtained in patient without gastro-esophageal acid regurgitation. Line A is pneumograph tracing and each downward deflection represents inspiration. Line B shows intraluminal pressure changes, scale on right. PIP is pressure inversion point: to left intraluminal inspiratory deflections are upward or intraabdominal; to right, they are downward or intrathoracic. pH changes are noted in Line C. Line D is signal marker and, reading from left to right, each mark represents removal of sensing device 1 cm upward from stomach toward esophagus. SW designates dry swallows. pH changes rapidly toward neutrality as PIP is passed.

FIG. 2. Record obtained on subject with gastro-esophageal acid regurgitation. pH remains low over area of several cm above PIP. See Fig. 1 for key.

method of making intraluminal pH measurements is comparable to that described by Rovelstad *et al.*(1,2) who discussed its limitations and advantages. For photokymographic recording, pressure transducer, pH meter, and pneumograph are attached to a Heiland multichannel oscillograph. This recording system is similar to that described by Bachrach *et al.*(3). Initially the tube is passed with subject sitting. Since inspiratory intrathoracic and intraabdominal pressures are, respectively, negative and positive, the location of diaphragmatic hiatus is readily determined by observing the level at which this reversal of deflections occurs. This point, as measured by distance of tips of tubes from nares, is reproducible to within 1 cm when tube is passed repeatedly both upward and downward. Fluoroscopic and X-ray examinations have confirmed that this point of pressure inversion is at the diaphragmatic hiatus. Subsequently, permanent photokymographic records are obtained by placing open tip of polyvinyl tube and the pH electrode in the stomach and withdrawing them 1 cm at a time or by passing them downward into the stomach in the same manner. Some patients manifest acid regurgitation when upright or supine; others, only when supine. Recordings, therefore, are made with subjects in the latter position. Acid regurgitation is diagnosed only when a pH of 3 or less is encountered over an area of at least 2 cm above the diaphragmatic hiatus, and when these observations can be reproduced by passing the sensing device in both directions several times. In practice this has not been a major problem, since the average length of acid regurgitation above the pressure inversion point among patients studied to date has been over 9 cm. However, it should be emphasized that variations in height of reflux in the same individual, tested at different times, are great.

Results. Fig. 1 illustrates a normal record. The tracing was started with sensing elements placed 3 cm below the hiatus. Thereafter, the tube was withdrawn one cm at a time. So long as it remained in the stomach, pH remained low and intraluminal inspiratory deflections were upward or positive. However, as pressure inversion point was passed, deflections became negative and pH changed rapidly toward neutrality. It is also apparent that intraesophageal pressures are lower than intragastric pressures, a finding that has been noted by all workers.

Fig. 2 illustrates acid reflux. The pH remained strongly acid for several cm above pressure inversion point, and only as the sensing device was removed far above the hiatus did it return to neutrality. A dry swallow had little effect on pH. In contrast, swallowing water (not shown) usually induced a rapid but transitory rise in pH which persisted for 5 to 30 seconds. On the recording, to right of pressure inversion point, there is a distinct increase in intraluminal pressure. Fyke, Code, and Schlegel(4) have recently described this phenomenon which they believe represents an intrinsic gastroesophageal sphincter. Similar zones have been noted in over half of our tracings, but no correlation between their existence and the presence or absence of acid regurgitation could be found.

To date, 90 subjects have been tested for evidence of gastroesophageal reflux, and acid regurgitation has been demonstrated in 54. Of this latter group, all but 2 had clinical, endoscopic, or radiographic evidence of esophageal inflammation. In contrast, no subject with normal records had either symptoms or findings referable to the esophagus. These findings lend support to the concept that regurgitation of gastric contents plays a dominant role in pathogenesis of esophagitis. In addition, it is felt that this new method will provide further information about the mechanisms whereby gastric contents are normally prevented from entering the esophagus, and the nature of the defect which permits such regurgitation.

Summary. Simultaneous intraluminal pressure and pH recordings at various levels within the esophagus and stomach were performed in 90 subjects. Evidence of esophageal inflammation was present in 52 of 54 individuals manifesting gastroesophageal acid regurgitation but was absent in all of those with normal records. A zone of increased pressure at the diaphragmatic hiatus could not be correlated with presence or absence of acid reflux. The evidence suggests that reflux of gastric contents is important in pathogenesis of esophagitis.

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Metabolic Inhibition Test for Determination of Antibodies to Group B Coxsackie Viruses.* (23999)

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The metabolic inhibition test of Salk, Youngner and Ward(1) has found extensive use in assay of poliovirus neutralizing antibodies. Several changes in technic of this test have been suggested(2-6), and recently Archetti *et al.*(7) have reported its use in the study of ECHO viruses(8). This paper outlines modifications of previously described procedures which permit determination of neutralizing antibodies for Group B Coxsackie viruses. The test has been evaluated by its performance in serodiagnosis of 55 cases of human infection with Group B Coxsackie viruses. Metabolic inhibition test depends upon appearance of demonstrable acidity as an indication of tissue culture cell viability. During these studies on Coxsackie viruses, Eagle's KB cell strain(9) was found preferable to monkey kidney cells as the host system.

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* Technical assistance was rendered by H. D. Huff and O. W. Dandridge.

Materials. Sera employed in evaluation of the test were obtained from 55 patients proved by appropriate studies to have Group B Coxsackie virus infections. In 41 of these cases the clinical syndrome was aseptic meningitis or "non-paralytic poliomyelitis." Most of remaining cases were diagnosed clinically as pleurodynia. The following materials were obtained from Microbiological Associates, Bethesda, Md.: 1) Medium A, lactalbumin hydrolysate and calf serum in Hanks' basic salt solution; 2) trypsinized first passage monkey kidney cells suspended in Medium A at concentration of 1,200,000 cells/ml; 3) trypsinized KB cells in Medium A supplemented with 10% horse serum, used at concentration of 100,000 cells/ml; 4) monkey kidney and KB cell stationary tube cultures; 5) Coxsackie virus strains B1, B2, B4 and B5, and 6) rabbit antisera to all 5 Coxsackie viruses. The Coxsackie B3 virus used was isolated in this laboratory from patient with pleurodynia. "Drakol," a heavy white mineral oil, was obtained from Pennsylvania Refining Co., Butler, Pa. Medium A with addition of 100 units of penicillin and 100 μg of streptomycin/ml was used as basic diluent for all tests. Stock virus suspensions were prepared from infected monkey kidney and KB cell cultures and maintained at −70°C between tests. *Methods.* The metabolic inhibition test as performed in this laboratory, utilizes sterile unstoppered Wasserman tubes in 90-hole wire serology racks. All sera are inactivated at 56°C for 30 minutes. To each of 5 replicate 2-fold dilutions of serum (1:10 through 1:640 distributed in 0.25 ml amounts), an equal amount of one of the 5 virus types is added. Virus suspensions are diluted to contain approximately 300 ID_{50}/0.25 ml. After incubation of the serum-virus mixture at room temperature for 1 hour, the KB (or monkey kidney) cell suspension is added in 0.25 ml amount to each tube. Controls for each test include: (1) tubes corresponding to 2 lowest dilutions of each serum incubated with cells but without virus to detect serum toxicity; (2) titration of standard rabbit antisera for each virus; and (3) serial 10-fold titrations of each stock virus, 5 tubes/dilution. Virus titer is calculated by the Reed-Muench formula(10). To obtain an approximation of metabolic activity of cells, 4 replicate 2-fold dilutions of the cell suspension are incubated without addition of virus or serum. Finally, each tube's contents are "sealed" with 0.5 ml of "Drakol." To maintain sterility each rack of tubes is covered with sterile aluminum foil at all times except during addition of reagents. Tests are incubated at 35°C until color changes are complete. Readings are usually taken after 5 and 7 or 6 and 8 days of incubation. Growth of virus is signaled by destruction of cells and preservation of red or alkaline color of the phenol red indicator in the medium. Absence of virus, or protection of cells from existing virus by antibody is made apparent by change in color of the indicator to yellow or acid form. Any detectable reddish or reddish-orange tint is considered indicative of virus action; when KB cells are used equivocal tubes can be jarred and observed macroscopically to determine whether cells are attached to the glass (alive) or free in the medium (killed).

Results. Virus titers: Table I summarizes

TABLE I. Range of Stock Virus Titers in KB Cell Metabolic Inhibition Tests.

Virus type	No. titrations	Titer variation*
B-1	13	7.8 to 8.5
2	13	6.7 7.7
3	13	6.8 7.8
4	15	6.7 7.7
5	13	6.9 8.1

* Expressed as the negative log ID_{50}/.25 ml.

TABLE II. Titer of Rabbit Antiserum for Type B-1 Coxsackie Virus in Tests Using KB Cells.

Date of test	Virus dose*	Serum titer†
4/25/57	2.6	1280
5/ 9	2.8	”
6/ 6	2.5	2560
13	2.5	”
27	2.5	”
7/11	2.6	”
25	2.8	”
8/22	3.0	”
29	2.7	”
9/12	2.5	”
10/ 3	2.5	”
7	2.4	”
11/ 7	2.2	1280

* Expressed as log index of test dose.

† Expressed as reciprocal of highest serum dilution causing neutralization.

data with respect to the reproducibility of the KB cell test. This Table shows range of titers obtained on serial testing of identical stock virus suspensions. The results obtained on 5 typical stock viruses, tested 13 to 15 times, are shown. In general, the titers from same virus did not vary by more than 1 log unit but in certain instances might exceed this slightly.

In addition, during the investigation it was noted that when KB cells were used the virus titers were consistently higher and more readily reproducible than when monkey kidney cell suspensions were used.

Serum titers. Table II displays the control rabbit antiserum titers in all tests performed to date with KB cells where B-1 Coxsackie virus dose fell between $10^{2.0}$ and $10^{3.0}$ ID_{50}. Similar results have been obtained with control antisera for the other 4 serotypes also, regardless of type of cell suspension used. At a given virus dose, serum titers are quite reproducible from test to test.

TABLE III. Effect of Coxsackie B-3 Virus Dose on Neutralizing Titer of a Human Convalescent Serum.

Test performed with KB cells		Test performed with monkey kidney cells	
Virus dose ($\log_{10}$ ID_{50} per .25 ml)	Serum titer*	Virus dose ($\log_{10}$ ID_{50} per .25 ml)	Serum titer*
.7	10,240		
1.3	5,120	1.5	>1,280
1.7	1,280	2.0	640
2.3	640	2.5	320
2.7	320	3.0	320
3.3	320		
3.7	160		
4.3	160		

* Expressed as reciprocal of highest serum dilution causing neutralization.

Table III shows the relation of a typical human convalescent serum titer to a varying virus dose in tests employing different cell types. While the same stock virus was used in both tests, it should be noted that only a limited comparison of serum titers was possible between the 2 systems, since the highest virus dose obtained in monkey kidney cells was only $10^{3.0}$ ID_{50}.

In Table IV the ability to demonstrate a significant (4-fold or greater) antibody increase with the metabolic inhibition test is correlated with the phase of disease during which the initial serum was drawn. It is apparent that the diagnostic efficiency of the test was impaired when the earliest serum available was drawn after the first week of disease.

TABLE IV. Coxsackie Virus Serodiagnosis: Importance of Early Collection of Acute Phase Serum.

Days after onset earliest serum drawn	No. of serum pairs	% successful diagnoses
0- 4	19	84
5- 7	9	78
8-14	26	46
14 or more	15	20

In 40 of the 55 cases tested the specific infecting Group B Coxsackie virus serotype was apparent, either because the agent had been isolated from stool, throat washings, or spinal fluid, or because there was antibody present for only one of the 5 serotypes. Thirty-three of these cases were examined for neutralizing antibodies by 3 separate technics, a metabolic inhibition test with monkey kidney cells, a metabolic inhibition test with KB cells, and a microscopic cytopathogenicity-neutralization test(11) using monkey kidney tissue culture tubes inoculated with a standard amount (approximately 300 ID_{50}) of the infecting virus. Results of all 3 methods were comparable, and neutralizing antibody increases were demonstrable with all 3 methods in 80-90% of the cases where the acute phase serum was drawn within 4 days of the onset of illness.

Heterologous antibody. As expected, the occurrence of antibody to other than the infecting Coxsackie virus serotype correlated with the age of the patient. Of the 6 preschool children tested, all had antibody to one type only. Heterologous antibody was present in only 3 of 10 cases aged 6 to 18 years in contrast to its presence in 16 of 24 patients over the age of 18. When heterologous antibody was present, a significant increase during the course of the infection occurred in 10 of the 24 older patients. In those cases with a significant increase in antibody to more than one serotype, from which virus could not be isolated, the specific infecting virus type could not be determined.

Discussion. The metabolic-inhibition test described has proved useful for the diagnosis of Group B Coxsackie virus infections. Laboratories already engaged in the study of poliomyelitis by this method will find no difficulty in adapting their procedure to this end. Eagle's KB cells were deemed more satisfactory than monkey kidney cells for this test on the basis of the higher and more reproducible virus titers obtained; either cell line gave satisfactory results in practice, however.

Other tests for Group B Coxsackie virus antibodies exist. A neutralization test in tissue culture tubes employing the microscopic observation of cytopathogenicity has been described(11). This procedure was used to corroborate the metabolic-inhibition test results obtained for this paper, but it is too time-consuming for routine application in the examination of large numbers of sera. Complement-fixation tests using antigens derived from infected suckling mice have also been described for this family of viruses(12-16). It is important to recognize, however, that problems relating to the occurrence of heterologous antibody increases may appear with this procedure(17) as well as with the neutralization test.

Data have been presented on the effect of the virus dose on serum titers. The logarithm of the serum titer is in inverse linear relation to the log dose but the data are insufficient to allow one to state categorically that the curve is straight line. Further observations will be necessary to establish the precise relationship.

Studies of normal human populations(18) have demonstrated that antibodies for Coxsackie viruses increase in prevalence with age. It is notable that in the present small series no children under 6 years of age possessed antibody for heterologous group B Coxsackie virus serotypes. Older children and adults frequently showed heterologous antibody and heterologous antibody increases. These data, like those obtained from the study of complement-fixing antibodies(17), support the hypothesis that increases of heterologous antibody for Coxsackie group B strains occur only in patients who have previously been infected with the heterologous serotype. There is no evidence that non-specificity plays a part in this phenomenon.

Summary. A metabolic inhibition test for detection of antibodies to 5 group B Coxsackie virus serotypes has been described. A serologic diagnosis could be made in as high as 84% of cases. Persons possessing neutralizing antibodies from previous group B Coxsackie virus infections frequently showed heterologous as well as homologous antibody increases.

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Colloidal Zirconyl Phosphate-P-32. Preparation and Tissue Distribution Following I. V. Administration. (24000)

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Radiocolloid therapy was first reported by Hahn and co-workers(1). Of the many radiocolloids employed since, radiogold and chromic phosphate-P-32 have been most widely accepted. Chromic phosphate is advantageous because of its pure, high-energy β-emanation as contrasted to the undesirable hard γ-ray from Au-198. Preparation of colloidal chromic phosphate has been achieved but proved time-consuming(2). Colloidal zirconyl phosphate-P-32, first described by Mayer and Morton(3), exhibits low solubility even in concentrated acids and is easy to prepare. However, as reported, the product is unstable and precipitates from neutral media.

This investigation was undertaken 1) to find a means of dispersing and stabilizing zirconyl phosphate colloid, 2) to investigate its fate in man and experimental animals, 3) to establish particle size, and 4) to determine its rate of excretion and radiation toxicity in experimental animals.

Methods. Preparation. By addition of zirconyl ion to aqueous orthophosphate a milk-white colloid is formed. In neutral media this sol is unstable and precipitates unless a suitable dispersant is added. Of the many substances tested for colloid stabilizing action, only 3 have merit: dextran, soluble starch, and glucose. Two of these, glucose and dextran, have been used routinely in the preparation of injectable zirconyl phosphate-P-32 sols. *Dextran-stabilized zirconyl phosphate*: 2 ml .1 M K_2HPO_4 was equilibrated with the desired amount of radiophosphorus[†] (100 mc P-32 in not more than 25 ml) for 10 minutes.

A drop of phenolphthalein indicator and 64 ml 6% dextran[‡] were added, and the solution neutralized with 1 N NaOH. After 5 minutes, 7 ml .1 N $ZrOCl_2$ was added dropwise with simultaneous adjustment of pH with NaOH. Volume made to 100 ml with saline. The colloid was then autoclaved for 20 minutes at 15 p.s.i. *Starch-stabilized zirconyl phosphate*: This procedure was similar to that for dextran-stabilized colloid. A 2.5% solution of soluble starch (Merck, according to Lintner) in .9% saline was centrifuged at 5,000 rpm for 20 minutes. Sixty ml of supernatant was employed for 100 ml of colloid. *Glucose-stabilized zirconyl phosphate*: 1) 2 ml .1 M K_2HPO_4 was equilibrated with 100 mc H_3PO_4-P-32 for 10 minutes. 2) 9 ml .1 M $ZrOCl_2$ was mixed with 50 ml 50% dextrose solution. 3) The equilibrium mixture, 1), was added slowly and with constant stirring to 2), followed by 3 rinsings of the container with 50% dextrose. 4) After 10 minutes the solution was titrated with 1 N NaOH to phenolphthalein end point. Volume was adjusted to 100 ml with distilled water, container sealed, and product pasteurized for 1 hour at 65°C. *Fate in Experimental Animals.* Studies were made of tissue localization of zirconyl phosphate-P-32 following intravenous administration in Swiss albino mice, Wistar albino rats, New Zealand white rabbits, and mongrel dogs. Tracer doses (20 to 50 μc/kg) suspended in dextran were administered to mice, rats, and dogs. Two rabbits were given lethal doses (1 mc/kg) of starch-stabilized colloid. Changes in the distribution pattern with time were studied in mice and rats by sacrificing groups of animals (2 or 3 animals/group) at varying intervals following injection. Tissues for radioassay were handled as follows: Animals were sacrificed with ether or sodium nembutal and the major organs removed and weighed. Duplicate samples (.05 to .20 g) of these tissues were introduced into weighed planchets, the weights determined, and incinerated at 500°C for several hours. This process facilitated radioassay by eliminating self-absorption correction. After ashing, radiophosphate content of each sample was determined with an end-window GM detector attached to model 172 Nuclear-Chicago scaling unit. Detector was calibrated with N.B.S. primary standards of P-32. *Excretion.* Total excretion per day of P-32 by the rat was investigated. Two animals were given 1 mc/kg of radiocolloid by vein, and thereafter daily collections of total excreta were obtained for 9 consecutive days. Each collection was homogenized, diluted to known volume, and assayed. *Radiation toxicity.* Toxicity of P-32 was studied in 8 male rats. Intravenous doses ranged from .1 to 2 mc/kg. Hematologic studies were made on 4 of these animals for 42 days. *Localization in Man.* Six patients with terminal acute leukemia and 1 patient with Hodgkin's disease were injected with small amounts of radiocolloid (.1 to 2 mc). In addition, 2 chronic leukemia patients, who had received the isotope therapeutically (4 to 7 mc), were included in this study. Seven of the 9 patients were given dextran-stabilized zirconyl phosphate and 2 were injected with glucose-stabilized preparations. In order to determine total radiocolloid content in the tissues studied, it was necessary that their total weights be known. Determination of gross weights of bone, bone marrow, lymphatics, muscle, gastro-intestinal tract, and blood was impracticable; so estimates were made on the assumption that a given tissue weight is proportional to total body weight(4). The tissues studied included most major organs and glands of the body. Those tissues of primary interest were liver, bone marrow, lymph nodes, spleen, lungs, and blood which are concerned with hematopoiesis, phagocytosis, and storage of the cellular entities of the blood.

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* From thesis in partial fulfilment of requirements for M. S. degree, Baylor Univ. Graduate Research Institute, Dallas, Texas.

[†] Available as H_3PO_4-P-32, carrier-free, from Oak Ridge National Laboratories, Oak Ridge, Tenn.

[‡] *Plavoflex*, 6% aqueous, Wyeth Labs.

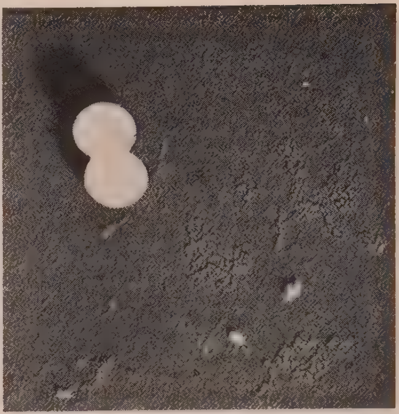

FIG. 1. Electron photomicrograph of preparation of zirconyl phosphate. Latex reference spheres .26 μ diameter.

Results. Zirconyl phosphate sols stabilized with dextran, starch, and dextrose are quite stable even after prolonged storage at 5°C. Dextran and starch-stabilized colloids can be autoclaved, but those stabilized with glucose must be pasteurized to avoid caramelization. When dialyzed against excess .9% saline, the preparations routinely contained less than 5% dialyzable P-32. Sterility of these preparations has been repeatedly demonstrated by thioglycollate culture methods. In no case was a pyrogenic response elicited when these products were administered intravenously to man and experimental animals.

An electron photomicrograph of a typical colloidal preparation is shown in Fig. 1. The average particle size has been estimated at .013 μ diameter. Uniformity and size of the colloidal particles were independent of suspending agent.

Tissue distribution data of P-32 following i.v. zirconyl phosphate-P-32 are presented in Table I. P-32 distribution appeared to reach equilibrium within a 5 hour period in mice

TABLE I. Tissue Distribution of P-32 in Experimental Animals following I.V. Zirconyl Phosphate-P-32.

	% recovered radioactivity			
	Rats	Mice	Rabbits	Dogs
Tissue	(Mean % ± S.D. of mean)*		(Mean %)†	
Liver	63.68 ±2.55	87.14 ±3.00	78.12	78.93
Spleen	30.56 ±2.47	4.39 ± .60	1.92	6.22
Blood	3.49 ±1.04	2.63 ±1.43	13.42	4.96
Kidneys	1.00 ± .18	1.27 ± .49	3.52	.70
Lungs	.62 ± .17	2.97 ± .96	2.13	.80
Heart	.31 ± .04	.52 ± .33	.87	.42
Testes	.33 ± .07	.10 ± .04		.06
Adrenals	.03 ± .003	.06 ± .01		.01

* 8 rats and 9 mice sacrificed between 5 hr and 14 days from inj. time (2 to 3 animals each at 5, 24, and 48 hr and 14 days).

† 2 animals each group. Dogs sacrificed at 3 days after inj.; rabbits at 10 days.

and rats and did not change appreciably thereafter. Concomitantly, blood levels of P-32 declined rapidly during this period, especially during the first hour. In the mouse, circulating P-32 was less than 5% of the total within 30 minutes after administration. The liver had the greatest capacity for radiocolloid accumulation in all species, exceeding 78% in mice, rabbits, and dogs. Total P-32 uptake by rat spleen was high compared to that of other species, though specific activity (μc P-32/g) of this tissue was high in all experimental animals. P-32 concentration in the bone marrow appeared to be high in the rabbit, minimal in the mouse and dog. All other tissues studied in the 4 species accounted for small increments of total recovered dose of radiocolloid. Accumulation in brain and testes was especially minute. In the mouse, specific activity of P-32 in bone was very low and did not increase with time. Total excretion of P-32 by the albino rat amounted to 1.11 ± .16%/day (Mean % ± S.D. of mean) of the total injected. These observations attest to the insolubility and chemical stability of colloidal zirconyl phosphate *in vivo*.

Radiation toxicity of zirconyl phosphate-P-32 in the rat was minimal. At the end of 8 months following injection, all animals were in excellent health and showed no evidence of permanent ill effects. The LD_{50} of zirconyl phosphate-P-32 is apparently greater than 2 mc/kg in the male albino rat (though 2 rabbits were killed by 1 mc/kg). Hematologic changes were characterized by a precipitous drop in leukocyte (primarily lymphocyte) concentration and a slight decrease in erythrocyte count. Hemoglobin concentration dropped in those animals receiving 2 mc/kg but did not change appreciably in the .1 mc/kg group. Variability in platelet counting was such as to obscure any significant change in this cellular component; however, no apparent thrombocytopenia developed.

In man, the primary sites of colloid accumulation were liver, bone marrow, spleen, muscle, lymph nodes, and lungs in that general order (Table II). These tissues contained between 85 and 96% of *recovered* radioactivity. Percentage recovery of *administered doses* of P-32 are listed at the bottom of Table II. Figures on each individual patient are given to show the pronounced variability of tissue uptake and to demonstrate the effect of time.

In terms of concentration of P-32 per gram of tissue, liver and spleen were comparable in most instances. Concentration in lymph nodes, bone marrow, adrenals, kidneys, and lungs decreased in that order. One patient (V.H.K.), who received glucose-suspended zirconyl phosphate, demonstrated an increased concentration of P-32 in the kidneys and diminished accumulation in bone marrow and lymphatics. The other patient (J.G.B.), who received a glucose-suspended preparation, demonstrated an uptake pattern not unlike those shown with dextran-suspended colloid.

Dextran-suspended zirconyl phosphate-P-32 has been employed successfully in the management of more than 100 cases of chronic leukemia and related blood dyscrasias at the Wadley Research Institute. Although this product was in general satisfactory, a few patients showed hypersensitivity to dextran; because of this, its use has been discontinued. Glucose-suspended zirconyl phosphate has proved to be completely satisfactory and is now used exclusively. The starch-suspended sols were not administered to human patients. Details of clinical evaluation of the products described above will be published elsewhere.

TABLE II. Tissue Distribution of P-32 in Leukemia and Hodgkin's Disease following I.V. Zirconyl Phosphate-P-32.

	% recovered radioactivity								
Tissue	J.Z.C.	J.G.B.*[h]	S.D.B.	V.H.K.*	O.C.B.	J.R.E.	W.J.D.	E.L.D.	E.W.
Liver	31.58	29.25	34.62	49.08	64.17	31.28	39.73	56.72	44.19
Bone marrow	35.08	19.72	10.32	1.64	7.18	18.64	13.16	18.22	7.48
Spleen	11.06	9.23	12.04	10.33	12.99	13.16	25.62	‡	15.27
Muscle	5.92	7.34	4.24	12.04	9.11	nm	10.19	12.96	20.41
Lymph nodes	5.02	2.53	3.99	.51	1.15	24.35	4.12	5.96	6.83
Lungs	6.10	3.75	19.83	4.18	1.22	6.85	2.11	2.34	1.52
Kidneys	3.39	2.54	5.54	11.84	.92	1.13	1.24	nm	1.33
Blood	nm	22.79	5.51	5.54	1.51	2.72	.77	.71	.69
G.I. tract	1.08	1.89	1.90	2.82	.95	nm	2.30	nm	
Bone	nm		1.10	nm				2.01	1.06
Heart	.42	.32	.35	.75	.25	.57	.36	.34	.45
Brain	.07	.19	.09	.27	.15	.71	.18	.12	.43
Pancreas	.13	.23	.16	.75	.23	.23	.17	.43	.21
Adrenals	.16	.17	.24	.23	.16	.11	.05	.13	.05
Thyroid	.02	nm	.02	.05	.01	.01	nm	.04	.07
Ovaries		.04			nm			nm	.01
Testes	nm		.02	nm		.07	nm		
Time†	2 hr	4 hr	12 hr	30 hr	3 days	6 days	8 days	10 days	22 days
Recovered dose (%)	47.3	61.3	61.2	51.7	97.5	80.8	117.5	114.8	84.1

* Given glucose-stabilized zirconyl phosphate. † Time elapsed from inj. to death of patient. ‡ Previous splenectomy.

[h] Hodgkin's disease. nm = not measured.

Summary. 1. Several technics of preparing stabilized sols of zirconyl phosphate-P-32 have been described using dextran, soluble starch, and glucose as suspending agents. All preparations exhibited good stability, small and uniform particle size, low solubility, and metabolic "inertness." High concentrations of glucose have proved superior to all other suspending agents for the preparation of injectable zirconyl phosphate sols. 2. In experimental animals, zirconyl phosphate-P-32 was concentrated rapidly in the liver, spleen, and bone marrow, when given intravenously. In the rat, a marked depression of the leukocyte count was effected. The LD_{50} for the rat was greater than 2 mc/kg but less than 1 mc/kg in the rabbit. Combined urinary and fecal excretion of P-32 by the rat was 1.11 ± .16%/day. 3. In human leukemia patients the radiocolloid was localized principally in liver, spleen, and bone marrow. Other tissues as muscle, brain, skeleton, and reproductive organs exhibited little affinity for the colloid. 4. This radiocolloid may be used to advantage in the rather selective irradiation of the reticulo-endothelial system in the management of chronic leukemia and related blood diseases.

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Structure and Function of Mitochondria in Irreversible Hemorrhagic Shock. II.* (24001)

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The first paper of this series cited reasons for our belief that the phenomenon of irreversibility in hemorrhagic shock was associated with defects in energy metabolism. Consequently, it seemed promising to study structural and functional state of mitochondria from dogs subjected to prolonged hypotension. Results reported in earlier papers have justified these assumptions: heart mitochondria from dogs in irreversible hemorrhagic shock yielded low P/O ratios and were altered morphologically(1). Furthermore, they did not undergo swelling and "crescent" formation as readily as normal controls under a variety of *in vitro* conditions(2). These findings suggested the possibility that, in shock, heart mitochondria acquired some unusual component or lost some normal constituent. The problem was approached by obtaining absorption spectra and chemical analyses on various kinds of mitochondrial extracts which differed in protein and coenzyme contents. The data to be reported here indicate that the alterations in composition associated with hemorrhagic shock in dogs are probably of a quantitative rather than a qualitative nature.

Methods. Procedures used for production of irreversible hemorrhagic shock in dogs and for isolation of heart mitochondria from experimental and paired control animals have been described(1). Briefly, dogs were bled and maintained at an arterial pressure of 40 mm Hg for 5 to 6 hours. Mitochondria were isolated by differential centrifugation in 0.38 M sucrose. The final pellets were suspended in 0.5 M sucrose. They were standardized turbidimetrically by diluting aliquots 1:100 in sucrose and reading the % transmittance at 680 mμ against water in a Coleman Universal Spectrophotometer. Each preparation was examined by phase contrast microscopy to ascertain its purity and structural integrity as judged by the absence of myofibrils and swollen mitochondria. *Severity of shock* was appraised routinely on the basis of (1) behaviour of the animal during the hypotensive period, particularly its ability to maintain the established hypotensive level; (2) degree of congestion and hemorrhage into the vital organs observed at necropsy; (3) extent to which mitochondria had undergone the morphological alterations characteristic of shock (1). *"Desoxycholate solutions"* of mitochondria were prepared by exposing the suspensions to 1% Na-desoxycholate. *Water extracts* were obtained by diluting the suspension with 5 or more volumes of distilled water, incubating in a 37°C water bath overnight and collecting the clear supernatants by centrifugation. For *perchloric acid extracts* the particles were exposed to 3 or 6% perchloric acid at 6°C overnight. After neutralization, volumes were adjusted (dil. 1:4 or 1:5) and supernatants collected by centrifugation. *Proteins* were determined by Gornall's method (3) in either desoxycholate solutions or water extracts. *Total nitrogen* was estimated by Johnson's method(4) or by titration after digestion and steam distillation of the ammonia formed into boric acid. *Phosphate* levels in the various supernatant fractions were determined essentially by the Fiske-Subbarow method(5) before and after a 15 minute hydrolysis in 1 N sulfuric acid in boiling water. Total pellet phosphate was estimated in the same way in the original suspensions after 5 hours' digestion with conc. sulfuric acid in the presence of a copper-selenite catalyst. *Absorption spectra* were determined using a Beckman DU Spectrophotometer. The data were *evaluated statistically* by applying the t-test to the differences between the individual pairs comprising each series of experiments.

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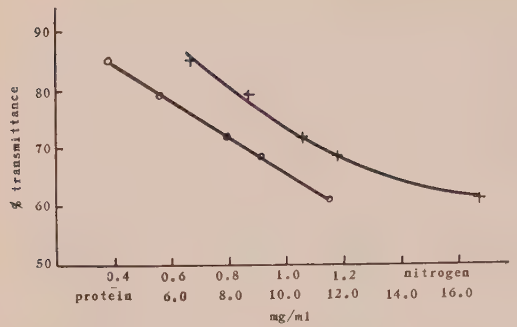
† Fellow of Am. Heart Assn.



A fresh mitochondrial suspension was diluted with varying amounts of 0.5 M sucrose

FIG. 1. Correlation of turbidity measurements with the protein and nitrogen contents of a suspension of dog heart mitochondria.

Results. Turbidity measurements may be taken as rough estimates of the number of particles/unit volume. This assumption is supported by the observation that, within a critical range, successive dilutions of a mitochondrial suspension yield protein and nitrogen values which vary approximately linearly with % transmittance (Fig. 1). In each experiment, therefore, the paired preparations from shocked and control dogs were adjusted to give the same turbidity reading in the general range of 72 to 80%. Their protein and nitrogen contents were usually found to be very similar and apparently unaffected by the severity of shock. In 25 experiments the average transmittance for both the normal and abnormal preparations was 77.5%, the average protein levels were 7.14 and 7.13 mg/ml respectively and the nitrogen contents 0.82 mg/ml for both.[‡]

This gross similarity of the 2 types of mitochondria was further borne out by the absorption spectra of desoxycholate solutions: one of 7 such pairs is given in Fig. 2a. These preparations were considered to represent more or less the whole mitochondrion in a dispersed, if not dissolved, state. They exhibited large protein peaks between 270 and 280 mμ and distinct Soret bands between 410 and 416 mμ, presumably mostly due to cytochromes. Absorption in the flavin and adenine regions was not pronounced.

In spite of this apparently close resemblance in protein and coenzyme contents, the total phosphate level in mitochondria derived from shocked dogs was abnormally high, especially so in cases of severe shock (Table I).

TABLE I. Total Pellet Phosphate in Heart Mitochondria from Normal and Shocked Dogs.

	μM/mg N		
Shock	Normal	Shocked	% increase
Slight	2.96	2.90	.0
	2.49	2.84	14.0
Moderate	2.16	2.54	17.6
	3.08	3.94	27.9
Severe	3.05	3.42	12.1
	2.64	3.09	17.0
	2.65	3.42	29.1
	2.94	4.24	44.2
	2.00	3.10	55.0
	$P < .003$		

These differences were found to be highly significant. Unfortunately, since these values were obtained after digestion, they yielded no clue as to the form in which this surplus phosphorus was present in the original particle.

Treatment of mitochondria with water left behind an insoluble residue of lipoidal and proteinatious material. Such extracts were, therefore, less concentrated than the desoxycholate solutions. Table II lists the percentages of the original protein and nitrogen recoverable in the aqueous supernatants. Fig. 2b gives a representative pair of spectra. Preparations derived from shocked dogs showed some tendency to be more water sol-

TABLE II. Percent Protein and Nitrogen Extracted into Water from Suspensions of Heart Mitochondria from Normal and Shocked Dogs.

% protein		% nitrogen	
Normal	Shocked	Normal	Shocked
23.2	59.0	14.8	44.8
14.4	14.4	37.9	27.5
17.3	23.8	29.6	47.0
19.8	29.5	29.1	54.7
30.6	27.1	35.6	50.8
17.7	22.9	20.2	10.8
18.5	29.2	16.5	7.9
22.0	23.5		
13.5	30.4	38.5	48.6
22.0	18.8		
		23.8	55.6
$P < .059$		$P < .084$	

[‡] For unknown reasons, however, daily variations were quite great, so that any particular transmittance value could not be correlated with any standard protein or nitrogen level.

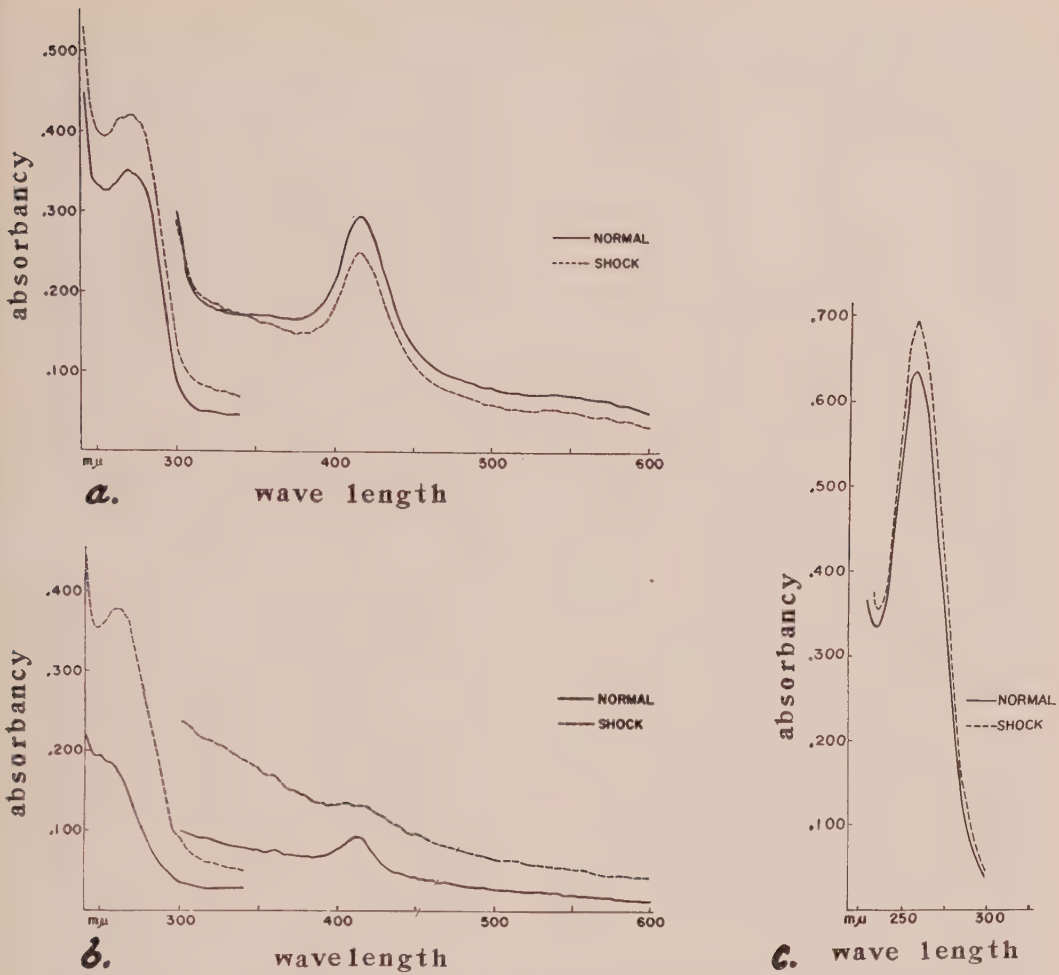


FIG. 2. Pairs of absorption spectra of heart mitochondria from normal and shocked dogs. a. Desoxycholate solutions: mitochondrial suspensions representing 0.94 mg/ml N (normal) and 1.08 mg/ml N (shocked) cleared in 1% Na-desoxycholate and diluted with water 1:5 (visual range) and 1:20 (U.V. range). b. Water extracts: normal = .32 mg/ml protein, .03 mg/ml N; shocked = .66 mg/ml protein, .08 mg/ml N. For U.V. spectrum the preparations were further diluted 1:3. c. Perchloric acid extracts: N-contents = app. 6-7 γ/ml.

uble. However, the extracted material did not appear to be grossly abnormal, for the pairs of absorption spectra became almost superimposable if reduced to the common denominator of 1 mg nitrogen. The peaks at 258 to 260 mμ corresponded to roughly 1 μM adenine/mg nitrogen. General absorption in the flavin region and the Soret band at 410 to 412 mμ also revealed nothing abnormal. The number of experiments was too small, however, to demonstrate any correlation between degree of solubility and severity of shock.

Perchloric acid extracts of mitochondria were free of the bulk of protein- and lipid-bound material and contained the dissociable purine and pyrimidine derivatives. The 2 types of mitochondria appeared very much alike under these conditions, both qualitatively and quantitatively. The extracts contained roughly 4% of the original nitrogen and exhibited clean peaks at 258 mμ (Fig. 2c). The spectra were not altered qualitatively when the mitochondria were preincubated in the presence of either the complete phosphorylating system or of the hexokinase-glucose trap alone.

Phosphate determinations run on these extracts revealed a tendency on the part of the

TABLE III. Phosphate Levels in Perchloric Acid Extracts of Heart Mitochondria from Normal and Shocked Dogs.

	μM/mg N of original suspension			
	Normal		Shocked	
	$P_{inorganic}$	$P_{acid\text{-}labile}$	$P_{inorganic}$	$P_{acid\text{-}labile}$
	.10	.04	.20	.01
	.20	.12	.23	.11
	.20	.18	.33	.18
	.19	.03	.20	.03
	.49	.28	.52	.31
	.17	.04	.30	.05
	.76	.37	1.16	.49
	.74	.11	1.27	.09
Avg	.36	.15	.53	.16
			$P < .042$	

Phosphates determined before ($= P_{inorganic}$) and after 15 min. hydrolysis in 1 N H_2SO_4 at 100°C. Diff. between the 2 determinations $= P_{acid\text{-}labile}$.

abnormal mitochondria to be richer than the normal in "inorganic" but not in acid-labile phosphate (Table III): Some of the surplus pellet phosphate apparently was present in the inorganic or a closely related form.

Discussion. On the whole, and within the limitations of the technic used, the spectral data suggest that heart mitochondria of shocked dogs do not contain any grossly abnormal constituent nor do they seem to lack any normal component. They tend to accumulate abnormal amounts of phosphate, some of it in a form which is extractable with perchloric acid and behaves like inorganic phosphate under Fiske-Subbarow conditions. Simultaneously, their proteins seem to undergo some physico-chemical alterations which result in a greater solubility in water. The protein fraction involved appears to contain bound flavins and possibly other substances absorbing at 260 mμ. This bound material is apparently not dissociable in perchloric acid.

The accumulation of phosphates within the mitochondria of shocked dogs complements our earlier observations on lowered P/O ratios. In view of the fact that some of this phosphate is in a form which reacts as inorganic phosphate under Fiske-Subbarow conditions, it is likely that the particles take up inorganic phosphate from the medium quite normally. The defect, therefore, appears to involve either the synthesis of ATP within the mitochondrion or the delivery of ATP to an external receiver system such as the hexokinase trap in Warburg experiments or the myofibrils in living muscle.

Summary. Heart mitochondria from dogs in irreversible hemorrhagic shock and from paired control animals were analyzed for protein, nitrogen and phosphate and their absorption spectra were compared. Similar analyses were done on water and perchloric acid extracts. The results indicate that, in shock, mitochondria accumulate abnormal amounts of phosphate, some of it in a form which is soluble in perchloric acid and reacts as inorganic phosphate under Fiske-Subbarow conditions. Simultaneously, some of the intramitochondrial proteins become more readily extractable with water.

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Barbiturate Potentiating Action of DDD and Perthane.* (24002)

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DDD (2,2-bis(parachlorophenyl)-1,1-dichloroethane) has been reported to cause adrenal cortical atrophy(1) species specific for the dog(1,2). In experiments involving surgical procedures on dogs previously fed technical grade DDD and anesthetized with barbiturates it has been noted that a high mortality results, regardless of the nature and extent of the surgery. Despite reports that production of adrenal cortical atrophy by DDD is species specific for the dog, attempts have been made to employ it(3-5) and Perthane(6), the ethyl derivative, (2,2-bis(paraethylphenyl)-1,1-dichloroethane) as an adrenal inhibiting agent in the human. Because of the possibility of a therapeutic compound related to DDD becoming available in the future it is deemed advisable to record this barbiturate hypnotic potentiating action of DDD.

Methods. Eighteen mongrel dogs of both sexes weighing 5-8.8 kg were arbitrarily divided into 4 groups, 2 groups of 5 and 2 groups of 4. During a control period all dogs were anesthetized by intravenous administration of sodium pentobarbital 40 mg/kg. Blood samples were drawn at times indicated in Table and barbiturate was determined by the method of Goldbaum(7). The endpoint of barbiturate action was taken as when the dogs were able to walk after being placed in an erect posture. Five dogs were given Tech. DDD 200 mg/kg/day. Because it has been shown that the most active principle(s) of Tech. DDD are contained in petroleum ether extractable mother liquors(8), a second group of 5 dogs received DDD recrystalized 8 times from petroleum ether. The dose was the same as in the first group. This recrystalized material consists almost entirely of p, p′-DDD. A third group of 4 dogs received the combined supernatant mother liquors from the preceding extractions, following evaporation of the extraction solvent, in daily doses of 50 mg/kg. This preparation contains, among possible other active principle(s) o,p′-DDD (9) which has previously been shown to cause adrenal atrophy(8). Perthane has also been reported to cause adrenal atrophy in the dog(10) so the fourth group of 4 dogs were given Tech. Perthane 200 mg/kg. All compounds were given orally in gelatin capsules once daily. Dogs in Groups I and II were anesthetized after 1, 10, and 14 days of feeding while dogs in Groups III and IV were anesthetized only after 14 days feeding. The results, excepting those at one day which showed no change from the control, are shown in Table I. An additional 2 dogs were dosed with Tech. DDD (200 mg/kg/day) for 14 days. Pentobarbital anesthesia was induced at the start and end of this period and blood samples drawn at 0, 15 minutes and 6 hours were analyzed for sugar and potassium. Since DDD does not cause histological changes in the rat adrenal it was deemed advisable to repeat portions of the experiment with this species. Thirty male Wistar rats weighing 200-230 g were used. Ten rats were given sodium pentobarbital 40 mg/kg intraperitoneally and the sleeping time noted. The rats were then fed 200 mg/kg of Tech. DDD dissolved in corn oil by stomach tube once daily for 2 weeks. Then the rats were again anesthetized and sleeping time noted. Another group of 10 rats were subjected to a different stress, namely anoxia. After being fed Tech. DDD in the same dose schedule as the preceding group of rats, these rats plus 10 controls were, over 4 minutes, taken to a simulated altitude of 25,000 feet and maintained there for an hour. A vacuum desiccator was used which permitted a continuous sweep of air through the chamber. Again on the following day the DDD fed animals and controls were subjected to a simulated alti-

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† A. D. Williams' Post Doctoral Research Fellow in Pathology.

TABLE I. Effect of DDD and Perthane on Duration of Anesthesia from Pentobarbital in Dogs and Rats and Blood Barbiturate Levels in Dogs.

		Control dogs, blood barbiturates in mg/100 ml blood					10 days feeding, blood barbiturates					14 days feeding, blood barbiturates					
Material	Wt (start)	15 min.	3 hr	6 hr	24 hr	Sleeping time (hr)	15 min.	3 hr	6 hr	24 hr	Sleeping time (hr)	15 min.	3 hr	6 hr	24 hr	Sleeping time (hr)	Wt (finish)
Tech. DDD	8.8	4.6	3.4	2.9	Nil	7	4.5	3.8	3.2	Nil	10	3.9	3.2	2.8	Nil	18	8.5
	6.7	4.3	3.7	3.0	”	7	3.7	3.4	2.5	”	26	4.1	3.7	3.3	”	36*	6.0
	5.5	4.1	2.5	2.2	”	8	3.9	3.4	2.9	”	12	3.8	2.9	2.6	”	36*	5.8
	8.5	4.1	3.6	3.4	”	7	4.2	3.8	3.3	”	10	Died†					8.0
	6.5	4.4	3.4	2.8	”	8	4.0	3.6	2.6	”	26	4.5	3.3	2.8	”	30	6.0
Recrystalized DDD	6.4	4.4	3.4	2.8	”	6	3.9	3.2	2.3	”	8	3.8	2.7	2.1	”	14	6.2
	8.5	3.4	2.4	2.0	”	8	4.1	3.5	2.3	”	10	3.8	3.4	2.5	”	16	8.0
	6.5	4.1	3.7	3.1	”	7	4.1	3.8	2.9	”	10	3.7	3.1	2.5	”	15	6.3
	6.0	3.4	2.5	1.9	”	6	3.9	2.5	1.9	”	10½	3.8	2.7	2.1	”	14	6.5
	5.7	2.5	2.0	1.7	”	6½	3.9	2.5	1.9	”	10	3.8	2.7	2.1	”	15	5.5
Mother liquor	8.0	3.7	3.3	2.4	”	6½						3.6	2.3	1.6	”	12	8.2
	8.0	4.7	3.2	2.4	”	6½						4.3	3.6	2.6	”	14	7.5
	5.0	3.9	2.6	1.5	”	7						3.7	3.4	2.7	”	24	5.2
	6.0	4.2	2.9	2.5	”	7						3.3	2.2	1.8	”	18	5.8
Tech. Perthane	7.5	4.1	2.3	2.0	”	6½						3.7	3.2	1.7	”	12	7.5
	6.5	3.4	2.4	1.7	”	6						3.8	3.2	1.9	”	12	6.2
	7.5	3.8	2.9	1.7	”	7						Clotted				14	7.5
	7.0	3.6	2.6	2.4	”	7						3.3	2.9	1.7	”	14	6.5
Tech. DDD	Rats (10)	218 ± 5 (g)				4.0 ± .1										4.2 ± .2	232 ± 8

* Sacrificed at 36 hr.

† Died immediately after inj. of anesthetic.

tude of 28,000 feet for 30 minutes. Both groups were immediately decompressed and sacrificed. The adrenals, pituitaries, livers, hypothalami of all dogs and rats were fixed in formalin, sectioned, and stained with hematoxylin and eosin.

Results. From the Table it can be seen that dogs fed Tech. DDD, ether extractable mother liquor from Tech. DDD, recrystalized DDD, and Tech. Perthane sleep longer after the same dose of barbiturates than before feeding with these materials. This effect is not apparent after the first day of feeding but is pronounced after 2 weeks of feeding. It appears that Tech. DDD is more potent in this respect than recrystalized DDD and Tech. Perthane. The effects of supernatant mother liquor are not strictly comparable because the dose used was one-fourth that of other compounds.

Microscopic examination of pituitaries and hypothalami of all dogs showed no unusual features, this is in agreement with early reports(1). Sections of adrenals from animals treated with Tech. DDD, mother liquor, and Tech. Perthane revealed usual atrophy which has been repeatedly described(1,10) and to about equal degree despite the fact that the dose of mother liquor was about one-fourth that of the other 2 compounds. Sections of adrenals from animals treated with recrystalized DDD showed no evidence of atrophy, in keeping with a previous paper(8). Sections of livers from all dogs revealed in each dog very minimal or no fatty change as contrasted with the more extensive fatty changes previously reported(1).

The fact that dogs receiving recrystalized DDD did not have atrophic adrenals but yet exhibited prolonged sleeping after barbiturate administration indicates that prolonged sleeping may not be mediated through the adrenals. It must, of course, be borne in mind that physiological functioning of the adrenal cannot be judged by histological appearance. The cause of this increased sleeping time is not apparent from these experiments. From the early report of liver damage by DDD(1), it was logical to presume that there may be delayed metabolism of administered barbiturates. However, this is not the case as is attested by the fact (Table I) that barbiturates are cleared from the blood stream with equal rapidity before and after feeding DDD. Finnegan *et al.*(11) have reported that DDD accumulates in fatty depots of the body, including the brain. Perhaps local changes in cellular metabolism of the brain not reflected in histological appearances may be the cause.

Lamson *et al.*(12,13) have shown that intravenous glucose and its metabolites such as lactate, among other substances, can prolong barbiturate anesthesia in some dogs. This action does not occur with lactate in the adrenalectomized guinea pig. Lasagna(14) has shown that K^+ also possesses this capacity. Despite the uncertainty in mode of action of the foregoing phenomenon it was decided to ascertain if blood sugar and K^+ were significantly affected by barbiturate anesthesia in DDD fed dogs. In 2 dogs fed DDD for 2 weeks and anesthetized with pentobarbital blood sugar and K^+ showed no significant deviations from similar analyses made prior to DDD feeding.

Administration of Tech. DDD to rats in same dosage as to dogs did not significantly prolong sleeping time under sodium pentobarbital (Table I). Since DDD does not produce adrenal cortical atrophy in the rat (1), this emphasizes another difference in the effects of DDD on the two species. In the anoxia tests applied to DDD-treated rats, a simulated altitude of 25,000 feet maintained for one hour had no untoward effect. However, a succeeding exposure to 28,000 feet for 30 minutes caused death of 5 of 10 DDD-treated rats but of none of 10 controls. It appears, therefore, that DDD in the dose used, in some fashion *slightly* lowers the threshold of the rat to anoxia. Sections of the organs from the rats showed no histological changes.

Conclusions. Tech. DDD, recrystalized DDD, supernatant mother liquor of Tech. DDD, and Tech. Perthane cause prolonged sleeping time in dogs given sodium pentobarbital. This increased sleeping time is not seemingly related to atrophy of adrenal cortex, and the very slight amount of fatty change in the liver does not cause prolonged clearance of barbiturates from the blood. This prolonged sleeping time is not obtained with similar doses of Tech. DDD in the rat although a *slight* lowering of tolerance to anoxia may result.

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Learning Deficit in Mature Rats Recovered from Early Post-Natal Infection With West Nile Virus. (24003)

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It has been established that certain viruses, which cause disease of the central nervous system (CNS) of man, can also cause a fatal CNS infection in rats, provided young enough animals are tested. A uniformly fatal encephalitis occurs in 7-day-old rats following either intracerebral injection or intranasal instillation of either St. Louis encephalitis virus (SLE) or Japanese B encephalitis virus (JBE). However, at 12 days of age rats manifest a high degree of resistance to both viruses and at 21 days of age are completely resistant, as far as clinically apparent infection is concerned(1,2). The resistance of older rats is not absolute, however, since, as has been demonstrated in the case of SLE, an inapparent infection occurs in 21-day-old rats (unpublished data). Furthermore, it has been observed that an occasional 12-day-old rat may develop a clinically apparent infection with JBE and then recover(2). When our studies were undertaken, the age-susceptibility pattern of the rat to West Nile virus (WN), a virus closely related to both SLE and JBE was being studied. It was found that the rat reacts to intracerebrally injected WN in the same manner that it does to JBE in that 7-day-old rats develop a uniformly fatal encephalitis while, in 12-day-old rats, some may succumb but most survive without evidence of CNS involvement although an occasional animal manifests signs indicating CNS dysfunction with subsequent recovery. On the other hand, rats 3 to 4 weeks of age never develop a clinically apparent infection. Although rats which have resisted or recovered from infection with above viruses do not subsequently show obvious abnormalities it seemed reasonable to assume that the viruses may have caused some damage to the CNS which could be reflected in subsequent behavior of the animal, especially behavior near the upper limit of complexity. To test this hypothesis one behavioral pattern, the learning of a moderately complex maze, was investigated. Rats which had received WN intracerebrally and rats which had received no virus, but whose brains had been similarly traumatized, were studied in a 14 choice-point modified Stone Multiple T-maze and the results compared.

Materials and methods. A single suspension of WN was used and was prepared by homogenizing WN-infected mouse brains of 3- to 4-week-old Rockland Swiss mice in a Waring blendor in sufficient inactivated, undiluted rabbit serum to make a 10% (W/V) suspension of virus-infected brains. The resulting homogenate was centrifuged 5 minutes at 3000 rpm and the supernatant fluid distributed in 2.2 ml amounts in screw-capped vials. The vials containing the virus were rolled in a dry-ice-alcohol bath and, after freezing, the virus was stored in a dry-ice cabinet. When needed, the virus was thawed by placing the virus-containing-vial in 37°C water bath. The mouse intracerebral LD_{50} titer(3) of the virus at the time it was used in the experiments reported here was $1 \times 10^{-7.7}$. A 10% (W/V) suspension of normal mouse brain (NMB), injected into control rats, was prepared in the same manner as described for preparation of the virus suspension. Sprague-Dawley rats, born on 2 consecutive days in animal quarters at the Medical Center, were injected intracerebrally with either 0.05 ml of the 10% suspension of WN or 0.05 ml of the 10% suspension of NMB, when they were 12 days old. Injections were made into the right cerebral hemisphere, using 0.25 ml syringe and 27 gauge ⅛ inch needle. No anesthetic was used. Some animals in each litter received WN and part NMB. Following inoculation, the animals were observed at least once, and frequently twice, daily for 21 days. They remained with the mother until they were 21 days old at which time they were weaned and sex determined. Males and females were kept in separate cages. When animals were 107 and 108 days old they were transferred from the Medical Center to North Little Rock Veterans Administration Hospital where one of us (ODM) conducted the maze studies. Prior to being transferred the rats were numbered by the method used by Walker(4). During maze learning studies the rats were tested by number and the experimenter had no knowledge of previous history of the animals. After approximately 1 month in their new quarters the animals were introduced to the maze. Before beginning any learning trials, the rats were placed in the goal box twice daily for 30 minutes on 5 consecutive days. Since thirst was to be used as motivation for running the maze, water was available to rats during 30 minute intervals spent in the goal box. After this period of orientation the animals were tested in the maze, one learning trial/day, for 8 consecutive days. The learning trials were then interrupted for 20 days after which they were resumed and continued for 7 consecutive days for a total of 15 trials. The 20-day interval was introduced to determine whether animals would forget, during this period, what had been learned during the first 8 trials. The time/trial and number of errors (number of blind alleys entered)/trial/rat were recorded. Test and control animals were deprived of water for 23½ hours prior to a learning trial.

Results. Twenty-four rats which had recovered from infection with WN and 18 NMB controls were run in the maze. The maze-learning curves of virus and control groups appear in Fig. 1 and 2. Fig. 1 shows mean

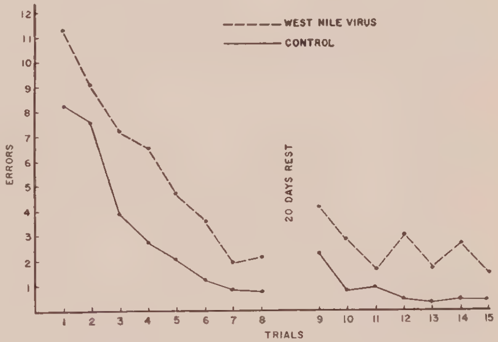

FIG. 1. Mean errors per trial.

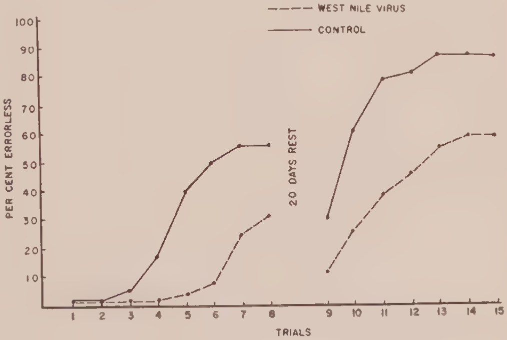

FIG. 2. % of rats having errorless run per trial.

TABLE I. Number of Rats Making Scores above and below Median.

	West Nile	Controls	Total
Above median	19	2	21
Below "	5	16	21
Total	24	18	42

errors/trial for each group and it will be noted that while the virus-infected animals did learn they did so at a slower rate than the controls. During the 20-day period between trials 8 and 9 the absolute memory loss of test animals is not noticeably different or at least not greater than that for the control group as shown by performances on 9th trial. Thereafter their learning proceeded at a slower rate and their performance never approached the level attained by controls. The percentage of each group achieving an errorless performance on a given trial is shown in Fig. 2. There is a striking difference in learning plateaus reached by each group. It will be noted that more than 50% of the controls had mastered the maze by the 7th trial while less than 30% of virus-treated rats had done so by the 8th trial. By the 13th trial approximately 90% of controls had errorless runs whereas no more than 60% of the WN had learned the maze after 15 trials.

Table I shows distribution of virus-infected and control rats above and below the median number of errors for all 42 animals. It will be seen that, while there is some overlapping, most infected rats had scores above the median whereas, control animals, with 2 exceptions, had scores below.

The data were subjected to further analysis by the Mann-Whitney U Test(5) and yielded a P-value of less than 0.01. This is a nonparametric test appropriate for distributions which are slightly skewed.

Discussion. Our studies were initiated to determine whether WN, injected directly into brains of 12-day-old rats, would affect their ability to learn a multiple T-maze. The results obtained indicate that the virus did influence learning ability. The shapes of the learning curves of virus-treated and control rats are quite similar but show that the control group learned more rapidly than the test animals. Following a period of rest between trials the WN group appeared to have proportionately no more memory loss than the control group. From these studies the cause of this reduction in ability to learn is not apparent. However, in studies not reported here, it has been observed that WN virus increases at least 10,000 fold in the whole brains of 12-day-old rats between 7 and 96 hours following intracerebral injection of the virus. If it could be demonstrated that certain nerve cells were preferentially damaged during this multiplication an explanation for the learning deficit might be evident. The rats used in the maze experiments seemed to possess good sensory functions, although it is possible that there might have been a subtle generalized sensory dysfunction. If the defect is on a sensory basis, it would seem that more than one sensory function would have to be involved since Honzik(6) has shown that the deficit following obliteration of a single sensory function is minimal. Since a rather difficult learning procedure was used, it is perhaps quite probable that the cause of impairment is referable to higher centers rather than sensory, unless it can be demonstrated that neuronal damage is so extensive that it involves combinations of tactual, visual, kinesthetic or olfactory pathways.

Summary. Our data indicate that West Nile virus, injected intracerebrally into 12-day-old rats, affects their ability to learn a moderately complex maze. After 13 learning trials approximately 90% of control animals had learned the maze, while no more than 60% of virus-infected animals had errorless runs after 15 trials. The memory loss of virus-treated animals following a period of 20 days between runs was proportionately no greater than that for controls.

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Salivary Gland Function in Rats. II. Effect of Thyroid Function on Salivary Flow and Viscosity.* (24004)

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Our previous studies have established a relationship between thyroid gland function and experimental dental caries in the rat(1,2). It has been postulated that the mechanism by which this phenomenon occurs is mediated through the salivary glands(3,4) although exact details are not clearly defined. Histologic changes in salivary glands occur when thyroid function is disturbed, and these changes have suggested the possibility of alteration in the physical-chemical function of salivary glands (5). The present studies were designed to help clarify the role of the thyroid gland in regulating salivary gland function and to provide some clue to specific alterations in function which might explain the dental caries changes.

Method. This study was divided into 3 series. Series I contained 18 adult male Sprague-Dawley strain rats. Group 1 of this series received 10 μg of sodium thyroxine subcutaneously each day, a second group received 0.1% propylthiouracil added to stock diet,‡ while group 3 were controls. The drugs were continued for 10 weeks when salivary flow was measured as follows: animals were anesthetized with nembutal (37 mg nembutal/kg body weight; concentration of solution was 5 mg/ml). Then, pilocarpine nitrate was given subcutaneously, 10 mg/kg of body weight. The volume of saliva, collected for 60 minutes, was determined by measuring in a calibrated syringe. Salivary viscosity was recorded as number of seconds required for saliva to pass between 2 marks 28 cm apart on finely drawn glass viscosimeter. Series II was composed of 2 groups. Group 1 consisted of 18 male weanling albino rats, 10 injected intraperitoneally with 500 μc of carrier-free radioactive iodine (I^{131}), while remaining 8 animals were controls. The base-line salivary flow and viscosity in radiothyroidectomized and control rats was determined after 90 days. Rats receiving I^{131} were given 10 μg of sodium thyroxine subcutaneously each day and salivary flow and viscosity again determined on third, fifth and ninth days following beginning of administration. Series III consisted of 63 male weanling rats divided into 5 groups: 1) controls; 2) animals thyroidectomized by administration of 600 μc of I^{131}; 3) radiothyroidectomized animals given 10 μg of sodium thyroxine daily; 4) radiothyroidectomized animals receiving 10 mg of testosterone§ intramuscularly every 3 days; and, 5) radiothyroidectomized animals receiving both thyroxine and testosterone at same levels as groups 3 and 4. Salivary flow and viscosity were measured one and 3 weeks following radiothyroidectomy and beginning of administration of thyroxine and testosterone. All animals were housed in raised screen cages and fed stock laboratory diet and distilled water *ad libitum*.

Results. The effect of thyroxine and thiouracil are seen in Table I, and indicate that thyroxine resulted in 33% increase in salivary flow as compared to 63% reduction by feeding propylthiouracil. The effect of thyroxine is not statistically significant ($p = 0.1$) although the effect of thiouracil is significant

TABLE I. Effect of Thyroxine and Thiouracil on Rat Salivary Flow (Series I).

Group	No. of animals	Avg body wt (g)	Salivary flow (ml/hr)
Control	4	360	2.9 ± .6*
Thyroxine	9	356	4.3 ± 1.2
Thiouracil	5	273	1.6 ± .4

* Stand. dev.

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* Supported by Office of Surgeon General, Dept. of Army.

† U.S.P.H.S. Post-Doctorate Fellow.

‡ Composition of diet as follows (in %): yellow corn grits, 52.7; ground yellow whole corn, 11.3; powdered whole milk, 30; alfalfa, 4.8; iodized sodium chloride, 1; and irradiated yeast 0.2.

§ Supplied generously by Schering Corp., Bloomfield, N. J.

TABLE II. Effect of Thyroxine on Salivary Flow and Viscosity in Radiothyroidectomized Rats (Series II).

Group	No. of animals	Avg body wt (g)	Salivary flow (ml/hr)	Salivary viscosity (sec.)
		0 days		
Control	8	267	1.6 ± .5*	54 ± 12
I^{131}	10	126	.1 ± .1	100 ± 26
		3 days		
Control	8	275	1.1 ±1.1	46 ± 7
I^{131} + thy†	10	123	.1 ± .1	86 ± 14
		5 days		
Control	8	257	2.6 ±1.0	58 ± 14
I^{131} + thy	10	126	.4 ± .3	73 ± 20
		9 days		
Control	7	260	1.8 ± .7	49 ± 7
I^{131} + thy	8	130	1.2 ± .8	49 ± 4

* Stand. dev. † thy = thyroxine.

$(p = 0.02)$.

Radiothyroidectomy resulted in significant reduction in salivary flow and significant increase in salivary viscosity (Table II). Thyroxine administered to thyroidectomized animals produced a gradual increase in salivary flow and reduction in salivary viscosity until, by end of ninth day, there were no significant differences between the 2 groups.

Table III shows data obtained in Series III. Radiothyroidectomy again resulted in reduction in salivary flow, thyroidectomized animals producing an average of 0.4 ml of saliva/hour as compared to 1.1 ml in controls after 3 weeks. Radiothyroidectomized animals receiving testosterone, thyroxine or combined, after 1 week showed no distinctive changes from I^{131} animals receiving no therapy. However, by end of 3 weeks, significant differences in salivary flow were found between controls and I^{131} animals. Animals receiving thyroxine, or thyroxine plus testosterone had a significant increase in salivary flow. Testosterone alone was partially effective in returning flow to the level of the control group. There were changes in salivary viscosity which generally paralleled flow but while directional, were not significant.

Discussion. Changes in salivary gland function, as evaluated by salivary flow and viscosity, parallel what might be expected to explain the effects of thyroid dysfunction on dental caries incidence. It is known, for example, that a marked reduction in salivary flow, brought about by extirpation of salivary glands, results in a significant increase in incidence of dental caries in the rat(6). Similarly, administration of I^{131} or propylthiouracil significantly increases dental caries(7). If the effects of these 2 agents are directly mediated through the salivary glands, causing a physiologic reduction in salivary flow, then one could readily explain the effect of thyroid gland function on dental caries on this basis. Our studies lend credence to this hypothesis by proving that thyroxine not only increases salivary flow, but that thiouracil and I^{131} reduce salivary flow. These observations correspond also with similar histologic changes suggestive of this effect(8,9).

Decreased salivary flow in Series I and II may conceivably be related to smaller body size of thiouracil-treated and radiothyroidectomized animals. This objection is not valid however, since animals in Series III which received thyroxine and testosterone weighed less than control animals yet produced far more saliva. The relationship of salivary flow to weight of salivary glands rather than body weight must await further study.

Summary. 1) We determined the effects of thyroid gland dysfunction on salivary flow and viscosity in the rat. Results indicate that

TABLE III. Effect of I^{131} Thyroidectomy and Replacement Therapy on Rat Salivary Flow and Viscosity (Series III).

Group	No. of animals	Avg body wt (g)	Salivary flow (ml/hr)	Salivary viscosity (sec.)
		1 wk		
Control	5		1.2 ± .8*	18.0 ± 4.9
I^{131}	8		.5 ± .6	17.1 ± 1.3
I^{131} + test.†	5		.5 ± .3	24.0 ± 6.9
I^{131} + thy.†	5		.9 ± .8	11.3 ± 1.0
I^{131} + thy. + test.	3		.5 ± .0	13.8 ± .3
		3 wk		
Control	4	166	1.1 ± .2	32.0 ± 8.8
I^{131}	5	120	.4 ± .3	41.4 ± 2.9
I^{131} + thy.	6	114	1.9 ± .9	28.5 ± 7.3
I^{131} + test.	4	123	.9 ± .5	40.9 ±10.0
I^{131} + thy. + test.	5	116	2.0 ± .6	27.9 ± 4.6

* Stand. dev.

† test. = testosterone; thy = thyroxine.

propylthiouracil and radiothyroidectomy reduced salivary flow and increased salivary viscosity. This can be reversed and function restored by administration of thyroxine. Testosterone partially restores salivary flow. 2) These data provide one explanation for the mechanism of alteration in dental caries incidence in the rat following disturbance in thyroid function, although the relationship is probably far more complex than this.

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Isolation of Tumor Cell Colonies in Tissue Culture.* (24005)

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Marcus, Puck, and Cieciura(1,2) described methods for isolation of "clones" of HeLa cells in petri dish tissue culture. They emphasized the necessity for the presence of more than a single cell to obtain growth. Goldstein(3) pointed out that it is not possible with this procedure to be certain that colonies originated from a single cell. He described a method for isolation of clones by which all but a single cell in a Carrel flask are destroyed by local heat with electric soldering iron. However, this cannot be done if single isolated cells fail to grow. The present study was made in an attempt to obtain colonies of cells which failed to grow singly and to avoid the difficulty in microscopic observation of cells grown in a petri dish. The ascitic form of a mouse ependymoma grown in A mice(4) previously could not be propagated from single cells isolated from droplets in a micropipet and injected subcutaneously in suckling mice (unpublished data). This procedure was successfully used elsewhere for growth of single tumor cells in mice(5,6). Likewise our attempts to grow single cells in a variety of media in capillary pipets(7) or on cover slips failed. These ependymoma cells are readily cultivated on glass in tissue culture. Isolated colonies originating from single cells were obtained as follows.

Materials and methods. After good growth of cells was obtained at 37°C in Kolle flasks containing a casein hydrolysate medium(8) with 10% horse serum, the cells were removed from the glass by contact at 37°C with 0.25% trypsin, pH 8, for 2-3 minutes. Serial dilutions of cells were made in chicken plasma, one drop placed near one end of each of 7-8 rectangular cover slips (11 x 32 mm) contained on moist filter paper in a small petri dish. An attempt was made to have 1-20 cells/drop after dilution. A second drop of plasma containing several hundred cells was placed near the other end of each cover slip. A drop of filtered chick embryo extract (CEE) was added first to the plasma drop containing diluted cells. The drop was mixed (with a fine glass rod bent to form an L) and spread

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over approximately one-half the area of cover slip. The other drop with many cells was then mixed with CEE and spread over the remainder of the slip. All slips were examined under a microscope to determine number and distribution of cells. Those which contained too many cells or cells not well separated from each other were discarded. After fibrin clots were formed, each slip was placed in a flat-sided (Leighton) test tube so that the cells were not in contact with the tube. Each tube then received 1.5-2 ml of above culture medium and closed with a rubber stopper and incubated. Cultures were observed under the microscope daily and culture fluid was changed twice weekly. Vigorous growth usually was observed after 2-3 days on portions of the slips heavily seeded with cells. Growth of individual cells on lightly seeded areas of the slip occurred in 4-7 days. After 14-20 days grossly visible colonies of cells could be seen. To transfer a single isolated colony, the cover slip was washed twice with fresh medium and removed from its tube to an open petri dish. The colony to be selected was located by use of dissecting microscope. A small piece of sterile tissue paper held with fine-pointed forceps was pressed against the colony and then placed in a drop of fresh plasma on another cover slip. After coagulation of the drop with CEE, the cover slip was placed in a fresh tube with culture medium and was incubated. Further subcultures were made directly onto glass without plasma. After several months the cells were routinely maintained in Eagle's medium with 10% horse serum. For mass production, cells were grown in 50 ml of medium contained in 32 oz. (1 liter) prescription bottles placed on a flat side. The virus used was a mouse brain tissue culture passaged strain of Theiler's GDVII mouse encephalomyelitis virus. Virus titer were assayed by hemagglutination with human rbc. Chromosomes of cells grown on cover slips were stained with 1% orcein in 50% acetic acid.

Results. Twenty-one colonies of cells were isolated of which 5 labeled A, O, R, U, and W were maintained and compared. The first 4 were indistinguishable morphologically. The W strain differed from the others in that cells were more definitely polygonal in shape and were more widely separated from each other when grown on glass. The cells also tended to spread out more and did not initially form closely packed colonies. Approximately 4% of cells in the original line were polyploid; none of the isolated colonies was. The A, O, and W strains grew at approximately the same rate, while the U cells gave the best growth and the R cells the poorest. During continuous growth the maximal cell counts attained were 3.7, 1.5 and 0.7 million per ml respectively for U, A and R strains. In a 32 oz bottle containing 50 ml of fluid approximately 100 mg wet wt. of cells were produced by the A strain.

The 5 strains of cells differed in their capacity to support growth of GDVII virus. Virus was grown in approximately equal numbers of cells for 3 days in Leighton tubes and the pooled supernatant fluid from 3 tubes was titered. Hemagglutination titers were as follows: A - 64, O - 256, R - 128, U - 32, W - 0, one day mouse brain - 1024. The W strain did not produce any virus.

The parent culture of cells from which the colony strains were isolated was obtained from ascitic fluid. It retained the capacity after 3 months, *in vitro*, to grow intraperitoneally in mice and to produce solid tumor on the serous membranes. After isolated colonies were maintained, *in vitro*, for 9-10 months, attempts were made to pass them intraperitoneally in mice by injection of each animal with 2-3 million cells in 1 ml. Mice examined 3 weeks later had growth of solid tumors on the serous membranes and 1-4 ml of ascitic fluid containing tumor cells with A, O, and R strains of tumor. W cells formed solid tumors but only a few drops of ascitic fluid. Mice examined 3 and 9 weeks after injection with the U strain of cells had no remaining tumor cells found.

Discussion. Practically, cell variations may occur so rapidly that strictly homologous cell collections will not be obtained. However, if the limitations of the procedure are recognized, useful information may be obtained from studies of variant cell populations. The derivation of cell variants can be a laborious task, particularly if individual isolated cells fail to grow. Growth of isolated cells in the environment of a nutritive colony as described here permits the development of colonies under direct observation. Detachment and wandering of cells is limited by the presence of plasma clots. Presumably each colony originated from a single cell. Because other cells are present in the culture it is not possible to be certain the colonies obtained are clones. At present there is no technic available which will obtain clones of cells which fail to grow as single isolated cells. In the meantime procedures of the type described here permit the derivation of cell populations which may differ considerably in regard to various characteristics.

Summary. A procedure is described for isolation of colonies of mammalian cells in tissue culture. Cell lines of a mouse ependymoma obtained in this way differed in growth characteristics, in capacity to grow, *in vivo,* and in susceptibility to Theiler's GDVII virus.

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Effect of Epinephrine on Adrenal Ascorbic Acid Following Premedication With Lysergic Acid Diethylamides or 5-Hydroxyindolalkylamines. (24006)

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Slight changes in molecular configuration of psychotomimetic drugs may alter their specific activity(1). Thus the interrelations between chemical structure and pharmacological properties of psychotomimetic analogs can be used as pharmacological tools to reveal biochemical mechanisms underlying behavioral changes induced by drugs. New findings have challenged the theories which propose serotonin antagonism as an explanatory clue for the mechanism of d-lysergic acid diethylamide (LSD) induced-psychosis(2). By comparing the pharmacology of d-2-brom LSD (BOL) and LSD it appears evident that increase of central sympathetic tonus following LSD injections fails to occur after BOL(1). It is, therefore, interesting to investigate the influence of various psychotomimetics on the actions of catecholamines on different neurohumoral mechanisms. Indolalkylamines are a class of chemicals including such psychotomimetic drugs as bufotenin(3). Lecomte demonstrated that serotonin sensitizes the nictitating membrane of the cat to epinephrine(4). Both bufotenin(5) and serotonin (6) can release epinephrine *in vivo* and the former is the more active compound. In contrast to bufotenin, serotonin does not cause hallucinatory experiences or behavioral changes. Thus, experiments were designed to follow changes in adrenal ascorbic acid content caused by epinephrine in rats premedicated with LSD, BOL, bufotenin and serotonin. In cats premedicated with indolalkylamines the variation of the contractive response of nictitating membrane to injected epinephrine was also investigated.

Method. Ascorbic acid was determined according to Roe and Kuether(7) on 350 Wistar rats decapitated 2 hours after intraperitoneal

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TABLE I. Adrenal Ascorbic Acid Content Two Hours following the Injections.

No. animals	Drug inj. intraper.	Dose (γ/kg)	mg ascorbic acid / 100 g adrenal tissue	Stand. error	% variation
20	Saline	—	423	407–440	0
8	Epinephrine	25	389	361–420	− 8
15	"	50	389	368–412	− 8
8	"	75	246	236–257*	−42
10	Bufotenin†	1000	330	285–355*	−22
6	"	500	395	338–452	− 7
8	"	250	456	431–481	+ 8
8	" + epin.	125 + 25	305	288–323*	−28
7	" + "	30 + 25	342	319–367*	−19
8	Serotonin‡	1000	313	284–341*	−26
8	"	500	338	308–368*	−20
8	"	125	447	433–462	+ 6
8	"	62	407	374–445	− 3
6	" + epin.	125 + 25	244	228–262*	−42
8	" + "	30 + 25	360	344–377*	−15
7	LSD§	200	309	293–327*	−27
7	"	100	381	366–397	−10
7	" + epin.	10 + 25	313	292–335*	−26
7	BOL§	400	430	411–451	+ 2
8	" + epin.	40 + 25	390	358–423	− 8

* $P \leq .05$.

† Bufotenin monooxalate H_2O (California Fn. for Biochemical Research, Los Angeles, Cal.).

‡ Serotonin creatinine sulfate (Nutritional Biochemicals Corp., Cleveland, O.).

§ BOL-148 and LSD-25 donated by Sandoz Pharmaceuticals, Hanover, N. J.

injections of drugs under investigation. The mean threshold doses evoking a superreactivity of cats nictitating membrane to injected epinephrine was considered the minimal quantity of a drug (γ/kg i.v.) causing a clear cut increase of the response of the nictitating membrane to threshold doses of epinephrine injected every two minutes. Furthermore our testing procedure required the increase response to appear 3 times during ten minutes following the injection of the drug. The cats were anesthetized with nembutal (15 mg/kg i.v.) and chloralose (40 mg/kg i.v.). For statistical analysis of results we used the Student's "t"-test and calculations were made according to Gaddum(8).

Results. In experiments reported in the Table both serotonin (0.5 mg/kg) and bufotenin (1 mg/kg) given intraperitoneally caused a significant depletion of adrenal ascorbic acid. Bufotenin, given in a dose of 0.5 mg/kg does not induce a decrease of adrenal ascorbic acid, and serotonin is therefore considered more active than bufotenin.

An ineffective dose of epinephrine (approximately ⅓ of the minimal effective dose) had been injected intraperitoneally with drugs under investigation. When ⅛ of the ineffective dose of bufotenin and ¼ of an equally ineffective dose of serotonin were injected with the epinephrine (25 γ/kg) a significant decrease ($P < .02$) of adrenal ascorbic acid was observed.

A more intense potentiation of the effects of epinephrine on the adrenal ascorbic acid content has been obtained by injecting greater doses of bufotenin and serotonin. A similar potentiation can be seen ($P < .001$) by combining small doses of LSD (10 γ/kg) with the standard dose of epinephrine. The decrease of the adrenal ascorbic acid content failed to occur after a higher dose (40 γ/kg) of BOL associated with 25 γ/kg of epinephrine.

Quantitative *in vivo* analysis(9) of the differences in the epinephrine potentiating effect of bufotenin and of serotonin was performed using the contractile response of the nictitating membrane of the cat. Applying this test to 10 cats we found that the threshold dose of bufotenin which potentiates the effects of epinephrine, was 4.82 γ/kg (3.41-6.81 S.E.) while that of serotonin was 88.01 γ/kg (57.57-134.50). We tried a similar *in vitro* analysis on the isolated ileum and spleen of the rabbit. But here neither bufotenin nor serotonin potentiated epinephrine. Both drugs, injected to cats in doses ranging from 25 to 150 γ/kg failed to facilitate the blood pressure effects of epinephrine. After cholinergic blockage (atropine 200-300 γ/kg) 25-75 γ/kg of bufotenin did not cause hypotension but hypertension. This hypertension did not occur following adrenalectomy or regitine premedication. While in normal cats bufotenin and serotonin evoked the same kinds of responses (hypotension followed by a small and delayed hypertension) after cholinergic blockage the serotonin effect was antagonized or curtailed while the one of bufotenin was reversed.

Discussion. Though LSD is more active than BOL in potentiating the adrenal ascorbic acid depletion evoked by epinephrine the mechanism of this potentiation is not understood. On a quantitative basis an inhibition of enzymes controlling epinephrine metabolism is improbable, in view of the fact that both potentiations can be obtained with very small doses. It is not clear whether, in the case of serotonin, the facilitation of cellular membrane permeability described by Woolley (10) can be proposed as an explanation for the epinephrine potentiation.

We do not believe that pharmacologically-induced hallucinations derive from a single mechanism such as inhibition of serotonin or sensitization toward epinephrine. Perhaps psychotomimetic effects result from more complex interactions. Nevertheless, these experiments demonstrate that epinephrine potentiation might be considered among the neurohumoral effects probably involved in the mechanism of the pharmacologically induced psychosis. The small doses of LSD necessary to potentiate epinephrine and the ineffectiveness of doses of BOL 4 times greater stress the importance of the parallelism between the intensity of the psychotomimetic effects of the 2 drugs in man and that of their interactions with epinephrine in rats.

The following considerations are added to emphasize the specificity of the above mentioned potentiation: though the LD_{50} of BOL (20 mg/kg i.v., in mice) is smaller than that of LSD (67 mg/kg i.v.)(11), the latter, on a weight basis, is more active in causing a depletion of adrenal ascorbic acid.

Thus a non-specific action cannot explain the inferior effectiveness of BOL. The potentiation of epinephrine caused by LSD could take place either by a peripheral mechanism (facilitating the adrenocortical action of ACTH) or by a central one (increasing the release of ACTH by epinephrine). The second hypothesis is more probable because LSD (50 γ/kg i.p. in guinea pigs)(12) fails to potentiate the effects of injected ACTH on the output of corticosteroids from the adrenal cortex. The results with 5-Hydroxyindolalkylamines suggest that epinephrine potentiation correlates psychotomimetic efficiency. The threshold dose of serotonin potentiating the epinephrine effects on nictitating membrane is greater ($P<.01$) than that of bufotenin. This parallelism is less evident in the adrenal ascorbic acid depletion test. Serotonin "per se" reduces more efficiently than bufotenin the adrenal ascorbic acid content; nevertheless, both drugs equally potentiate epinephrine effects on adrenal glands. Finally, in agreement with previous reports(5) our results support the possibility that bufotenin releases catecholamines from adrenal medulla.

Summary. 10 γ/kg i.p. of LSD potentiate the adrenal ascorbic acid depletion caused in rats by 25 γ/kg i.p. of epinephrine, 40 γ/kg i.p. of BOL are ineffective in this respect. 30 γ/kg i.p. of both serotonin and bufotenin potentiate the effects of epinephrine (25 γ/kg i.p.). The threshold doses of bufotenin and serotonin enhancing the epinephrine induced contractions of cat's nictitating membrane were 4.82 γ/kg i.v. and 88.01 γ/kg i.v.

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Effect of Reserpine Phosphate on Dividing Onion Root-tip Cells. (24007)

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Goldin *et al.*(1) and Belkin and Hardy(2) have shown that large doses of reserpine, administered to mice carrying lymphoid tumor L 1210 or solid tumor Sarcoma 37, increase survival time and cause tumor regression. Induction of a deeply tranquilized state in animals reported by these authors and noted in our experiments with reserpine, inferred that antitumor activity may be the consequence of prolonged depression rather than an intrinsical and specific antitumor action of the drug. To determine more closely the mode of antitumor action of reserpine, the effects of this alkaloid in various biological systems were investigated. Initial microbiological experiments on *in vitro* activity of reserpine phosphate indicated that it does not act directly on bacteria; no influence on cell multiplication rates was recognized. However, certain colonial modifications and morphological distortions of various gram positive and gram negative cells were observed. Swelling, chain formation, and pigment inhibition were in large measure dependent upon stage of development of the cells concerned. As with many other agents, dividing cells were more vulnerable than resting stages. It was interesting, therefore, to investigate the effect of reserpine on mitosis in higher plants. This was done using root-tip cells of *Allium cepa* var. Silver Skin.

Methods. "Silver Skin" resting bulbs were germinated by placing bulb on top of shell vials of 10-20 ml capacity filled daily with freshly distilled water. The vials were coated with thin layer of paraffin to exclude light. After 3-4 days, bulbs with 15 or more roots were selected for testing. These bulbs were then transferred to similar vials containing 0.125 to 10 mg% reserpine phosphate. The solutions were changed daily for duration of experiment. To obtain as much variation in cell counts as possible, roots from 4 different bulbs were used for each dilution of compounds tested and for controls. Root tips, carefully removed at 24, 48, and 72 hours, were fixed in acetic-alcohol, squashed, and stained with aceto-carmine according to method described by Belling(3). Control bulbs were germinated in water with and without sodium phosphate, smeared, and stained in the same manner as test roots. To reduce deviation and inconsistency, roots were collected at same time each day (1 p.m.)—a time reported by Kellicott(4) to be a period of maximum mitotic division in *Allium* root-tips showing diurnal variation. Totals of at least 1000 cells in the region of cell multiplication were counted for each sample, and the number of cells in mitosis from late prophase to telophase recorded. Countings were made after one, 2, and 3 days in reserpine and after second, third, and fourth day replacement in water. Conclusions reported below are based on determinations of mitotic index and analyses of dividing cell populations in terms of phase percentage. The mitotic index is a ration of dividing to resting cells.

Results. Table I shows mitotic index values from roots exposed 24 hours to various dilutions of reserpine. At concentration of 4 mg%, the compound exerted an irreversible toxic effect. Within the 24 hour period, at this and higher concentrations, mitotic division was absent; roots presented a flaccid condition from which no recovery was possible even though the roots were placed in fresh water for 4 or more days following reserpine treatment. Arrest was obvious in the late prophase or early metaphase stages. At 2 mg%, the compound lacked toxic properties and at lower concentrations an increase in metaphase as well as total mitotic division was apparent. Increase in mitotic index was greatest at concentration of 0.5 mg%, (Table I). Variation in number of cells in metaphase was correspondingly lower and at 0.25 mg% reached a maximum of 38% more than the controls. It would seem, therefore, that reserpine does not affect the initiating mechanisms of mitosis, nor of the mechanisms of mitosis itself.

For controls, approximately 19,000 cells of 14 untreated roots were counted. The majority (96.44%) were in the resting stage, 1.72% in metaphase, 0.74% in anaphase, 0.61% in prophase, and 0.56% in telophase. Similar values were recorded during daily examination periods throughout the experiments and with sodium phosphate used in comparative studies.

When the counts for different periods of exposure were arranged chronologically (Table II), it becomes apparent that roots exposed to 0.5 mg% reserpine contain a gradually increasing percentage of cells in mitosis and in metaphase reaching a maximum at 24 and 48 hours respectively and declining after 72 hours to a point near the figure observed for untreated root tips. It is interesting to speculate that this difference is due to accelerated growth development in the presence of the compound at specific concentrations. Reserpine phosphate increases mitosis by increasing prophase and metaphase stages.

TABLE I. Mitotic Index of Allium Root-Tip Cells Exposed 24 Hours to Reserpine.

mg %	Total mitosis	Metaphase
	%	
4	.63	.37
2	3.81	1.77
1.5	3.97	1.98
1	4.62	1.99
.5	5.93	2.10
.25	5.22	2.37
.125	4.57	2.10
.0 (control)	3.66	1.72

TABLE II. Mitotic Index of Root-Tip Cells Exposed to .5 mg % Reserpine.

Hr of exposure	Total mitosis	Metaphase
	%	
0	3.66	1.72
6	5.40	1.80
24	5.93	2.10
48	4.91	2.37
72	4.35	1.66

After 2 and 3 days growth in 2 mg% reserpine solutions (renewed every day), several roots showed vague swelling and distortion. Such roots replaced in water resumed normal growth, leaving the swollen or distorted area behind. Slight thickenings were also observed in root growth of radish seeds exposed to higher concentration of the alkaloid during seed germination studies. Although these macroscopic observations suggested similarities to the activity of colchicine as described by Levan(5), alteration of chromosome activity was not apparent in these swollen parts, upon microscopic examination. Neither were the colchicine-like effect of spindle solution or clumped condensed chromosomes—the latter condition following retarded prophase noted by Dustin(6) with certain "Radiomimetic" compounds—observed with reserpine treated roots. It may thus be presumed that the action of reserpine phosphate is different from that of colchicine.

Summary. The effect of reserpine phosphate on dividing onion root-tip cells showed that this alkaloid was toxic at 4 mg%. In lower concentration an increase in mitotic index was noted. A maximum ratio of dividing to resting cells was observed at 0.5 mg% after 24 hours exposure to drug. Although an intensification in metaphase was present, the compound was not capable of arresting the metaphase. Thus, reserpine is not a mitotic inhibitor of all cells. If it is possible to draw conclusions from these experiments upon the effect of reserpine on mouse tumors, one would think that regression of mouse tumor under high alkaloid medication is not due to an intrinsic anti-tumor action of the drug.

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Serum Protein and Glycoprotein Alteration in Swine with Experimental Arthritis.* (24008)

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A naturally occurring inflammatory arthritis of swine which frequently persists following systemic infection by *Erysipelothrix rhusiopathiae* has been described(1-4). A consistent finding in patients with active rheumatoid arthritis is an increased concentration of carbohydrate-rich globulins (serum glycoproteins)(5). Serum glycoprotein levels have been utilized as objective means of evaluating current inflammatory activity of patients with rheumatoid arthritis(6,7). The present investigation is concerned with the study of serum protein and glycoprotein changes in swine following the production of chronic arthritis by injections of *Erysipelothrix rhusiopathiae.*

Method. The incidence of arthritis following acute infection by this organism can be substantially increased by first vaccinating the swine with bacterins and later administering an intravenous challenging dose of *E. rhusiopathiae.* Under such circumstances approximately 90% of surviving animals develop the characteristic inflammatory arthritis(4). Swine of both sexes weighing approximately 200 lb were used. After intravenous injection of 10 ml of a 24 hour culture of *Erysipelothrix rhusiopathiae* in semi-solid medium, all animals developed acute swine erysipelas with high fever followed by death of approximately half of the animals. Initial blood samples were taken from 16 surviving

TABLE I. Effects of Swine Arthritis and Acute Erysipelas Infection on Protein-Bound Carbohydrates and Proteins of Swine.

	Arthritic			Initial infection
	Normal	Inactive	Active	
No. of animals in group	11	4	12	16
Total serum glycoprotein (mg %)*	163 ± 6	162	229 ± 5 †	205 ± 4 †
Seromucoid fraction (mg %)*	12 ± .7	16	31 ± 1.6 †	29 ± 1.9 †
Total serum protein (g %)	7.17 ± .11	7.10	7.47 ± .12†	7.23 ± .09 †
(Total serum glycoprotein + total protein) × 100	2.27 ± .06	2.28	3.10 ± .06†	2.85 ± .06 †
[(Total serum glycoprotein-seromucoid) + total protein] × 100	2.10 ± .05	2.04	2.65 ± .04†	2.45 ± .03 †
Seromucoid + total glycoprotein	.075 ± .002	.099	.136 ± .006	.138 ± .007†

* Expressed as bound hexose (galactose-mannose).
† Significantly higher than normal value at 1% level of probability.

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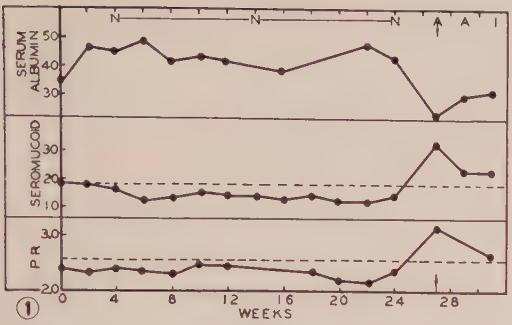

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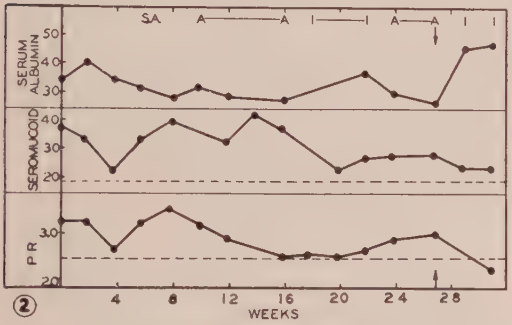

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FIG. 1. Serial studies of an arthritic swine. Animal recovered from initial inflammatory stage and then developed acute arthritis at 27th wk, indicated by letter "A" at top of chart; N indicates lack of clinical signs of arthritis. Note increase of seromucoid and PR (serum glycoprotein : protein ratio) and decrease of serum albumin during the acute phase. Serum albumin is expressed as % of total serum protein, seromucoid, as mg of hexose/100 ml of serum, and PR, as g of bound hexose/100 g of total serum protein.

FIG. 2. Serial studies of an arthritic swine. Animal recovered from acute inflammation but rapidly developed arthritis (as indicated by A——A at top of chart). At 18 wk, remission occurred as indicated by I——I. Note decrease in seromucoid and PR (glycoprotein hexose : protein ratio) during this period, followed by exacerbation of the disease on 24th wk. On 27th wk this animal was treated with cortisone with clinical improvement. Serum albumin is expressed as % of total serum protein, seromucoid, as mg hexose/100 ml of serum, and PR, as g of bound hexose/100 g of total serum protein.

animals 17 days post exposure, and comparisons were made to sera of 10 unexposed control swine. The body temperature of exposed animals returned to normal 3-8 days before the sampling. Subsequent samples were taken every 2 weeks for 31 weeks. Total serum glycoprotein hexose was determined by the tryptophan method of Shetlar, Foster, and Everett(8), and serum protein by the biuret method. Seromucoid was isolated by the method of Weimer and Moshin(9) and quantitated as hexose by the tryptophan method. Paper electrophoretic studies were made as previously described(7) using 3 MM filter paper and the Spinco hanging strip electrophoresis apparatus.

Results. Serum glycoprotein and protein concentrations in normal and arthritic swine in the active phase are summarized in Table I. The effects of the initial acute erysipelas infection on serum glycoprotein and protein as found on the first bleeding are also shown, as are findings in those arthritic animals whose disease had become clinically inactive. Serum glycoprotein and seromucoid levels were significantly elevated in all animals following erysipelas infection. Similar elevations were also noted in those animals which developed chronic arthritis after the initial phase of the disease. Serum glycoproteins remained significantly elevated even after that portion due to seromucoid was deducted from the total glycoprotein bound hexose. However, it is noteworthy that the ratio of seromucoid to total serum glycoprotein was increased significantly by acute erysipelas, as well as by the ensuing chronic arthritis. Swine with inactive arthritis exhibited serum glycoprotein and seromucoid concentrations within the range normal for swine.

Results of serial studies of 2 swine are graphically shown in Fig. 1 and 2. Fig. 1 summarizes data of animals which appeared to recover from the initial infection and then developed acute arthritis during the 27th week of study. When the clinical condition improved, a decrease in serum glycoproteins and an increase in serum albumin was noted. Fig. 2 illustrates data concerning an animal which recovered from early acute inflammation, then rapidly developed arthritic symptoms. Cortisone treatment (300 mg/day) was started on the 27th week producing clinical benefit, paralleled by a decrease in serum glycoproteins and an increase in serum albumin as described above.

Definite α_1-globulin peaks were not noted in any of the swine paper electrophoretic patterns. This is in agreement with electrophoretic patterns of pig serum obtained by Deutsch(10) using a Tiselius technic.

TABLE II. Summary of Changes in Glycoprotein Hexose and in Serum Protein following Administration of Cortisone to Arthritic Swine.

	Before cortisone	After cortisone
Glycoprotein	Bound hexose as mg/100 ml	
Total	202	174
Seromucoid	21	15
α-globulin	95	89
β- "	48	39
γ- "	49	31
Albumin	11	14
Protein	Protein as g/100 ml	
Total	7.48	8.02
α-globulin	1.26	1.30
β- "	1.38	1.21
γ- "	2.58	1.58
Albumin	2.26	3.94

This absence of α_1-globulin in the arthritic swine is particularly interesting as this fraction exhibits striking changes in the human patient with active rheumatoid arthritis. The γ-globulin was found to be elevated (compared to the human) even in patterns of control swine. After cortisone treatment the γ-globulin fraction was reduced below that of the control animal. Glycoprotein electrophoretic studies of the sera of the various groups indicated that elevations of bound carbohydrate attached to the α-, β- and γ-globulin fractions occur in the acutely arthritic animals. A decrease of these components occurs after cortisone administration (Table II), being particularly evident in the γ-globulin and seromucoid fractions. No change occurs in the α-globulin fraction if the assumption is made that all of the seromucoid is found in the α-globulin after electrophoresis.

Summary. A serial study was made of protein and glycoprotein changes of swine during arthritis which follows recovery from *Erysipelothrix* infections. As in sera from humans with rheumatoid arthritis, elevated serum glycoprotein, seromucoid and serum globulins and lowered serum albumin concentrations were noted in swine sera during active phases of the disease. No serum α_1-globulin was identified in swine sera. During remissions of experimental arthritis, serum glycoprotein levels decreased and the serum albumin increased. Cortisone treatment of animals with active arthritis produced clinical improvement accompanied by striking increases of albumin protein with decreases of seromucoid, serum γ-globulin protein and γ-globulin bound hexose concentrations.

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Tumor Cell-Induced Mouse Ascites Fluid as a Source of Viral Antibodies. (24009)

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In studying the immune response of mice to viral antigens, there are severe limitations to the volume of blood serum obtainable. As one possible solution to this problem Munoz (1) reported that considerable quantities of mouse peritoneal fluid result from injections of Freund's adjuvant and that this fluid is rich in specific antibodies induced by injections of certain albumins. The present report will show that Sarcoma 180 cells are also convenient for inducing large volumes of ascites and that such peritoneal fluid contains virus-induced antibodies. Wagner(2) has stated that passively immunized mice exhibit viral antibody levels in Ehrlich tumor cell-induced ascites similar to those found in the blood serum. This report will show that a similar result can be obtained with Sarcoma 180 cell-induced ascites produced in mice actively immunized with viral antigens.

Materials and methods. The viruses used were chick embryo adapted lines (American Type Culture Collection) of the PR8 strain of influenza A virus and the California strain (#11914) of Newcastle disease virus (NDV). A single allantoic fluid pool of each of these viruses was used for immunizing procedures as well as for subsequent hemagglutination-inhibition tests. Groups of 20 Swiss mice (Webster strain, 18 to 20 g) were immunized by injecting into the peritoneal cavity 0.2 ml of undiluted allantoic fluid pools of either NDV or influenza virus. These injections were repeated twice weekly every other week for 6 weeks. Immediately after the last immunizing injection, all mice were injected intraperitoneally with 0.5 ml of a 1/20 balanced salt solution dilution of S-180 cells obtained from a mouse exhibiting marked abdominal distention resulting from prior injection of tumor cells. In these experiments each mouse received approximately 1 million tumor cells, although the number of such cells implanted does not seem to be critical. When the mice became markedly swollen by accumulation of peritoneal fluid (in 7-10 days), they were anesthetized with ether and heart blood as well as ascites were collected separately. Since it was not known what effect clotting might have on antibody content of the ascites, heparin was used to prevent clotting of blood and of ascites. In that antibody content of blood was to be compared to that of the ascites, it was necessary that a uniform volume to volume ratio of these 2 fluids be maintained during their collection. For example, in a group of 20 mice, one mouse could have a high antibody titer, while another might have a low one. Collection of a comparatively large volume of blood and a small volume of ascites from the mouse with the high antibody titer and the converse with the low titered mouse, would lead to a serum sampling error when all blood samples from a single group were pooled. Thus, it was arbitrarily decided that for each 0.1 ml of heart blood obtained from a mouse, 1 ml of ascites would also be collected from the same mouse. Blood from all mice in a single group was combined and centrifuged at 2000 rpm for ½ hour to remove the cells. A similar procedure was followed for the ascites samples. Both fluids were ampuled and stored at dry-ice temperatures. The above procedure can be complicated by the unpredictable death of mice that received S-180 cells. This problem was largely overcome, however, by use of large groups of animals. For example, in a total of 40 mice only 8 died before they could be utilized. This disadvantage may be further counterbalanced by using pools of fluids from large groups of mice, where the results cannot easily be influenced by an unusual response of a single animal. Eventually, of course, all mice will die over a period of time that depends on the number of S-180 cells

TABLE I. Hemagglutination-Inhibition of Immune Mouse Blood Plasma and Ascites Fluid against Newcas Disease and Influenza A Viruses.

Antibody source	Immunizing virus	Test virus	Dilutions of plasma or ascites fluid (Exp. I)									Red c contr
			1/80	1/160	1/320	1/640	1/1280	1/2560				
Blood plasma	Influenza	Influenza	—	—	—	—	+	+				—
Ascites		”	—	—	—	±	+	+				—
”		NDV	+	+	+	+	+	+				—
Blood plasma (2 tests)	NDV	NDV	—	—	+	+	+	+				—
Ascites (2 tests)		”	—	—	+	+	+	+				—
Ascites		Influenza	—	+	+	+	+	+				—
			Dilutions of plasma or ascites fluid (Exp. II)									
			1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	
Unheated ascites	Influenza	Influenza	—	—	—	—	—	+	+	+	+	—
Heated ” *		”	—	—	—	—	—	+	+	+	+	—
Unheated ”		NDV	+	+	+	+	+	+	+	+	+	—
Heated ”		”	+	+	+	+	+	+	+	+	+	—
Unheated blood plasma		Influenza	—	—	—	—	—	—	+	+	+	—
Heated ” ”		”	—	—	—	—	—	±	+	+	+	—
Unheated ” ”		NDV	+	+	+	+	+	+	+	+	+	—
Heated ” ”		”	+	+	+	+	+	+	+	+	+	—
Unheated ascites	NDV	NDV	—	—	—	—	+	+	+	+	+	—
Heated ”		”	—	—	—	—	+	+	+	+	+	—
Unheated ”		Influenza	—	+	+	+	+	+	+	+	+	—
Heated ”		”	±	+	+	+	+	+	+	+	+	—
Unheated blood plasma		NDV		—	—	—	—	+	+	+	+	—
Heated ” ”		”	—	—	—	—	+	+	+	+	+	—
Unheated ” ”		Influenza		+	+	+	+	+	+	+	+	—
Heated ” ”		”	—	±	+	+	+	+	+	+	+	—

* 56°C for 30 min.
+ = complete hemagglutination; ± = partial hemagglutination; — = no hemagglutination.

originally implanted. When 1 million cells were implanted, the average day of death for a group of 10 mice was 14. The level of viral antibody in blood plasma and ascites was determined by hemagglutination-inhibition procedure(3). It was first necessary to titrate both virus pools to determine the highest saline dilution that would completely agglutinate a 2% suspension of washed, chicken erythrocytes. Based on this, a standard virus suspension was prepared so that 1 ml contained 8 times more virus than necessary for complete hemagglutination. The antibody-containing fluid to be tested was diluted in 2-fold steps in 0.85% NaCl solution. The standard virus suspension was mixed with each plasma dilution in a 1:1 volume ratio. The antibody-antigen mixtures were incubated 1 hour at room temperature and 1 ml of each mixture was added to 0.5 ml of a 2% suspension of red blood cells in a plastic depression plate, which was rotated vigorously to insure mixing of the reagents. The resultant pattern of the agglutinated erythrocytes was read after 45 min.(3).

Results. The data (Table I) indicate that ascites contains antibodies in amounts which are comparable to those found in blood plasma. There seems little doubt as to the specificity of the influenza virus neutralizing antibodies, since essentially no non-specific neutralization of NDV was observed. This was not true, however, in one portion of Exp. I, where dilute NDV-specific ascites appeared to neutralize influenza virus. In this experiment neither the ascites nor blood plasma was heated to remove heat-labile non-specific hemagglutination-inhibitors. Exp. II (Table I), however, resolved this point, since heated (56°C for 30 min.) as well as unheated ascites were tested. In this experiment, non-specific inhibitors were not observed at dilutions greater than 1/16. It can also be noted that the antibody titers observed in the first

TABLE II. Neutralization of Infectivity of Influenza A (PR8) Virus for Mice with Specific Immune S-180 Induced Ascites.

Dilution of ascites	Survivors* / Total mice
1/ 80	4/5
1/ 160	5/5
1/ 320	5/5
1/ 640	2/5
1/1280	5/5
1/2560	4/5
Control	0/5

* Experiment was terminated 14 days after infection.

experiment were higher than those in the second. There is no explanation at present for this result other than there may have been differences between the chicken red blood cells used.

Additional preliminary data (Table II) indicate that dilute, specific immune peritoneal fluid will neutralize the infectivity of influenza A virus for mice. In this experiment, a mouse lung-adapted strain of influenza A virus was diluted so that 0.1 ml contained 100 LD_{50}'s. The virus suspension was then mixed with dilutions of the ascites in a 1:1 volume ratio and incubated at room temperature for 1 hr. The antibody-antigen mixtures were then instilled intranasally in 0.1 ml volumes into the mice. Control mice received virus diluted 1:1 with saline. The data show that the peritoneal fluid neutralized the infectivity of the virus at a dilution greater than 1/2560. It is apparent that when a small amount of virus is used, as in this experiment, the antibody titer of the ascites is very high. Other experiments indicate that specific immune ascites for influenza B (LEE) virus will also neutralize the interfering capacity of this virus.

Discussion. It is a simple matter to obtain large quantities of ascites from mice, injected with S-180 cells. It is frequently possible to obtain 10 ml from a single mouse, and not unusual to collect 15 and 20 ml. In our experiments, all mice accumulated significant amounts of peritoneal fluid and an average of 5 ml was conveniently obtained from surviving mice. This amounted to at least 10 times as much antibody as could be obtained by cardiac puncture. Thus, it would seem desirable, in certain cases, to use mice in place of those larger animals now maintained only for occasional use as an antibody source.

Mice that have been injected with S-180 cells frequently contain significant volumes of ascites over protracted periods of time. This would suggest the possibility of infecting the mice with a virus and then following the antibody rise by periodic intraperitoneal tappings.

Summary. Mice immunized by multiple injections of either influenza A or Newcastle disease virus produced antibody levels in the Sarcoma 180 cell-induced ascites comparable to levels found in blood plasma as measured by hemagglutination-inhibition test. The infectivity and interfering capacity of influenza viruses could also be neutralized by the specific immune ascites. It was possible to collect at least 10 times more ascites than heart blood.

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Correlation of Intimal Sudanophilia and Cholesterol Analyses of 3 Layers of Human Aorta.*† (24010)

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The purpose of this report is to present the the results of cholesterol analyses of 189 samples from a single human aorta which had been stained *in toto* with a fat stain, Sudan IV. Other investigators(1,2,3) have performed chemical analyses including cholesterol determinations on lesions of atherosclerosis, but there has been no attempt, as far as we can ascertain, to correlate intimal sudanophilia with cholesterol concentration in the manner to be described.

This study was undertaken to determine degree of correlation between sudanophilia and cholesterol concentration in human atherosclerosis and whether or not there is a gradient of cholesterol concentration in any of the aorta's 3 dimensions. Since fat stains such as Sudan IV are used for selective staining of atherosclerotic lesions in humans(4-7), and the lesions resembling atherosclerosis in experimental animals(8,9); and since cholesterol is a constant component of atherosclerotic lesions, a study of this nature seemed indicated.

Materials and methods. A single aorta was stained grossly with Sudan IV and divided into 189 blocks. Cholesterol analyses were performed on these blocks. The aorta was obtained 3 hours post mortem from a 25 year old white male who died an accidental death. This case was selected to minimize any effect of terminal illness on aortic lipids. The vessel was opened anteriorly, stripped of adventitial fat, flattened on chipboard, and fixed 24 hours in 10% neutral formalin. Following fixation the entire aorta was stained with Sudan IV (saturated solution of Sudan IV in equal parts of acetone and 70% ethanol) for 15 minutes, differentiated in 80% ethanol for 20 minutes and washed in tap water for one hour. The staining solution, differentiating solution, and wash waters were retained for cholesterol analysis. Fig. 1A depicts the aorta after staining with Sudan IV. Prior to staining there were noted scattered pale yellow streaks and spots on the intimal surface of the aorta, which were most prominent in the abdominal portion. They stained red with Sudan IV and appear black in the photograph. There were no fibrous plaques or complicated lesions (ulcerated, thrombosed, or calcified) present. Fig. 1A illustrates the 63 pieces into which the vessel was initially divided. Each of these pieces was placed flat on a freezing microtome with the intima up-

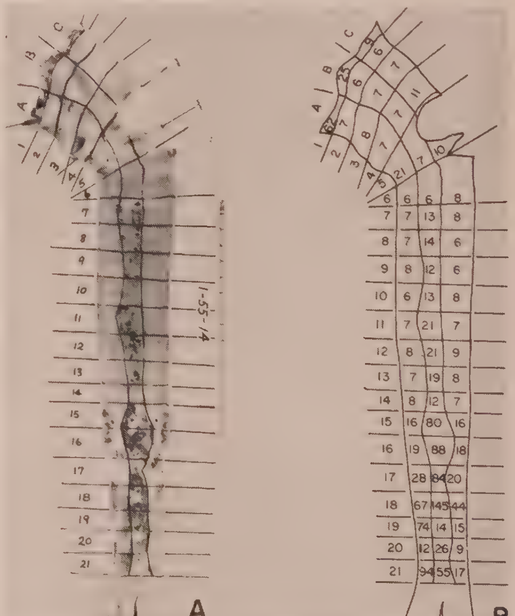

FIG. 1. A. Intimal surface of aorta showing areas of Sudan staining and the 63 pieces into which aorta was initially divided. B. Cholesterol values of intimal layer of aorta expressed to nearest whole number.

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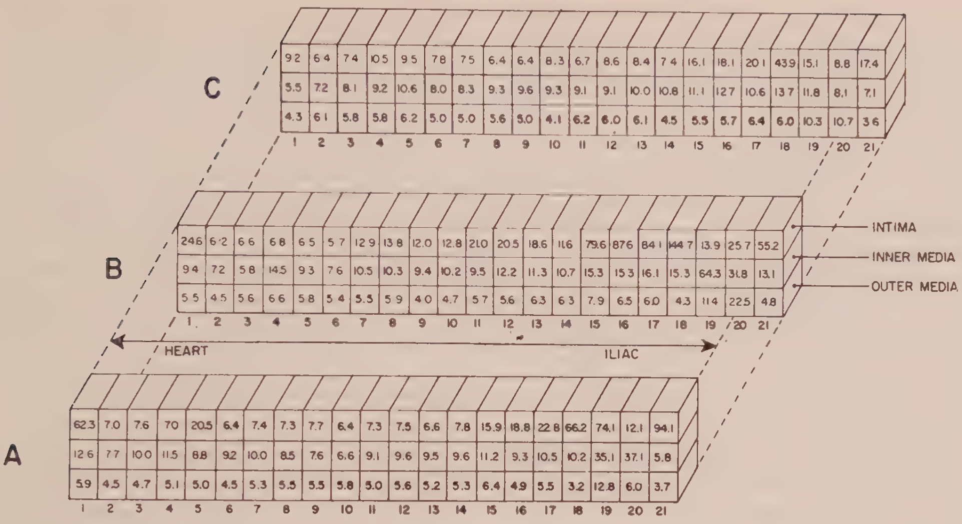

C

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
INTIMA	9.2	6.4	7.4	10.5	9.5	7.8	7.5	6.4	6.4	8.3	6.7	8.6	8.4	7.4	16.1	18.1	20.1	43.9	15.1	8.8	17.4
INNER MEDIA	5.5	7.2	8.1	9.2	10.6	8.0	8.3	9.3	9.6	9.3	9.1	9.1	10.0	10.8	11.1	12.7	10.6	13.7	11.8	8.1	7.1
OUTER MEDIA	4.3	6.1	5.8	5.8	6.2	5.0	5.0	5.6	5.0	4.1	6.2	6.0	6.1	4.5	5.5	5.7	6.4	6.0	10.3	10.7	3.6

B

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
INTIMA	24.6	6.2	6.6	6.8	6.5	5.7	12.9	13.8	12.0	12.8	21.0	20.5	18.6	11.6	79.6	87.6	84.1	144.7	13.9	25.7	55.2
INNER MEDIA	9.4	7.2	5.8	14.5	9.3	7.6	10.5	10.3	9.4	10.2	9.5	12.2	11.3	10.7	15.3	15.3	16.1	15.3	64.3	31.8	13.1
OUTER MEDIA	5.5	4.5	5.6	6.6	5.8	5.4	5.5	5.9	4.0	4.7	5.7	5.6	6.3	6.3	7.9	6.5	6.0	4.3	11.4	22.5	4.8

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
INTIMA	62.3	7.0	7.6	7.0	20.5	6.4	7.4	7.3	7.7	6.4	7.3	7.5	6.6	7.8	15.9	18.8	22.8	66.2	74.1	12.1	94.1
INNER MEDIA	12.6	7.7	10.0	11.5	8.8	9.2	10.0	8.5	7.6	6.6	9.1	9.6	9.5	9.6	11.2	9.3	10.5	10.2	35.1	37.1	5.8
OUTER MEDIA	5.9	4.5	4.7	5.1	5.0	4.5	5.3	5.5	5.5	5.8	5.0	5.6	5.2	5.3	6.4	4.9	5.5	3.2	12.8	6.0	3.7

Cholesterol Concentration in Human Aorta

Milligrams cholesterol per gram of dried defatted tissue

FIG. 2. Exploded block diagram of aorta with cholesterol values expressed as mg/g of dry defatted tissue for intima, inner media and outer media.

permost and quick frozen. A thickness of approximately 160 M was sliced from the intimal surface. This thickness was selected because it was found that this removed the stained portions of the intima from the media in deeply stained areas. The remaining media was divided into 2 approximately equal portions and referred to as inner media and outer media. This yielded 189 specimens for cholesterol analysis. Each sample of tissue was extracted with Delsal's solution(10) with 5 changes of solvent. The first, second, fourth, and fifth changes were decanted after 5 minutes. The third solvent change was allowed to stand 12 hours. The combined solvent extracts were evaporated at 60°C under nitrogen and the lipids re-dissolved in 1:1 acetone-alcohol for a single determination of cholesterol by the Sperry-Webb(11) procedure. The residual tissue was dried at 60°C *in vacuo*, placed under desiccation and weighed repeatedly until a constant weight was obtained. The total amount of cholesterol remaining in the dried defatted tissue was determined by hydrolysing the combined tissues with 30% potassium hydroxide for 16 hours at 100°C, extracting the hydrolysate with ether, and analysing in-replicate for cholesterol according to the Sperry-Webb procedure.

Results. Fig. 2 depicts in an exploded block diagram of the aorta the values for cholesterol expressed as mg cholesterol/g of dried defatted tissue. Fig. 1B shows cholesterol values of the intimal layer for comparison to the photograph of the stained aorta (Fig. 1A). The stained intima has a distinctly higher cholesterol concentration than the unstained intima. The unstained intimal values vary from 6 to 9 mg cholesterol/g of dried defatted tissue, and the stained intimal values vary from 12 to 145 mg with higher values from blocks with more intense and extensive Sudan staining. Values in the inner media and outer media are relatively constant with the inner media averaging 11.7 and the outer media averaging 5.9 mg cholesterol/g of dried defatted tissue. Medial specimens showing widest deviation from usual range of values have penetrating blood vessels in or immediately adjacent to the block.

Analysis of the Sudan IV stain, decolorizing alcohol, and wash waters for cholesterol revealed that 5.6% of total cholesterol recovered from the vessel was removed in the process of staining. Of this 5.6% cholesterol removed in the staining process, 4% was removed in the Sudan IV staining solution, 1.6% in decolorizing alcohols, and none in wash waters. Analysis of the dried defatted tissues by alkali digestion revealed that 0.45 mg or 0.8% of total cholesterol recovered remained in the tissue.

Discussion. Studies of early lesions of atherosclerosis in the human aorta have shown that gross Sudan staining of the entire aorta facilitates detection of early lesions(6). Sudan IV stains all lipid substances except those with high melting points. Cholesterol, excluding low melting point esters, is not stained by this dye(12). Since increased amounts of cholesterol are present in atherosclerotic lesions we were desirous of knowing the relationship between cholesterol concentration and sudanophilia.

This study shows that the extent and intensity of Sudan staining is directly proportional to cholesterol concentration even though cholesterol does not stain with Sudan. This indicates that cholesterol when present in increased amounts is rather constantly associated with other lipids, such as triglycerides, which do stain with Sudan. Areas of the intima, completely free of any staining with Sudan IV, ranged in value from 6 to 10 mg cholesterol/g of dried defatted tissue while those areas that stained with Sudan ranged from 12 to 145 mg cholesterol. Block 19B is the single notable exception to the rule having a cholesterol value of 14 mg and taken from a heavily stained area. This discrepancy is probably due to incomplete removal of the intimal layer since the underlying media has a cholesterol concentration much higher than the mean for that area.

With little exception the cholesterol concentration of the inner and outer layers of the media is quite constant. The values for the inner media lying beneath stained areas are in general higher than that beneath unstained areas which probably reflects incomplete removal of the fatty deposit. Consideration of medial values and unstained intimal values gives no evidence of a gradient of cholesterol concentration along the length of the aorta for man as described in experimental animals (13). In the unstained areas the intimal value is greater than for the outer media, and both of these values are usually less than the value for the inner media.

The staining process removed 5.6% of total cholesterol from the aorta. This is a rather small amount but it must be remembered that this 5.6% probably came first from the surfaces of the vessel in closest contact with the solvents, and principally from those areas containing the largest amounts of cholesterol. It is possible that the generally lower level of cholesterol concentration in the outer media is related to this rather than being a truly lower value.

Summary. A single aorta from a 25-year-old white male was stained grossly with Sudan IV and divided into 189 specimens for cholesterol analysis. The results show that the extent and intensity of Sudan staining is directly proportional to cholesterol concentration in the tissue. Analysis of the data reveals no evidence for a gradient in cholesterol concentration along the length of the aorta. The cholesterol value of the inner media is generally higher than either the intimal or outer media cholesterol concentration in areas where no lipid deposit is contained in the intima. The cholesterol concentration in the outer media is consistently lower than the value for the intima or inner media.

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Immunologic Significance of the Cell Wall of Mycobacteria. (24011)

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Separation of bacterial cells into their major morphological elements (cell wall and internal protoplasm) by a method first described by Salton and Horne(1) offers a new approach to antigenic analysis of bacteria. When aqueous suspensions of bacteria are vibrated with minute glass beads, the rigid cell wall cracks, and protoplasm is liberated in a dispersed state. Cell walls are then separated from protoplasm by differential centrifugation. Examination of a fungus and several bacteria by this technic showed that important antigens are situated in the cell wall(2-6).

Whereas the procedure of Salton and Horne is directly applicable for fractionation of certain bacterial species, it cannot be successfully applied to mycobacteria unless special measures and precautions are taken. To achieve selective disruption of mycobacteria, suspensions of washed cells must be monodispersed when exposed to mechanical vibration, and products of disruption must also be maintained in a dispersed state to allow final separation of cell wall from protoplasm.

Methods. *Mycobacterium butyricum* and 2 strains of *M. tuberculosis* (BCG and H37 Ra) were studied. *M. butyricum* was grown at 37°C for either 3 days on solid Meadow agar medium(7) or for 24 hours in liquid Meadow medium. BCG and H37Ra cultures were grown in stationary liquid Dubos Tween-albumin medium(8) for 8 to 10 days at 37°C. Bacterial cells were collected and washed by centrifugation in cold water containing 0.5% Tween. Washed bacilli could be adequately redispersed in aqueous Tween. *M. tuberculosis* grown on solid or liquid media in which granular growth occurred did not disperse adequately in Tween solution. All procedures described below were performed at 4°C. Suspensions of dispersed washed bacteria were diluted with Tween solution to a concentration having an optical density of 700 mμ in a Klett-Summerson nephelometer with filter No. 420. Five ml of suspension mixed with 5 g Cataphote No. 12 glass beads were shaken in the Mickle tissue disintegrator for 10-second periods for a total shaking time of 4 minutes. Agitation was discontinued between each of these periods for 10 to 30 seconds to avoid raising the temperature of the suspension. About half the cells were broken after this mechanical treatment. It must be emphasized that products of the Mickle process have a marked tendency to clump during vibration and prolonged treatment will produce aggregates to such an extent that cell walls cannot be obtained. Under the experimental conditions employed, a vibration period of 4 minutes was usually suitable. However, the optimum vibration time for different lots of cell suspension must be individually determined by electron microscopy of samples taken from the Mickle tubes. Difficulties regarding aggregation were not encountered during mechanical disintegration of other bacteria.* The mixture of nondispersible aggregates, intact cells, cell walls, and protoplasm was allowed to stand until the glass beads settled. The suspension was then decanted into conical 12-ml tubes, centrifuged, and supernatants containing protoplasm were removed. All centrifugations were performed at 2,000 g for 60 minutes. The sediment in each tube was washed twice by thorough resuspension in Tween solution and centrifugation, then resuspended in Tween solution and allowed to stand overnight or until the aggregates settled. Following centrifugation of resultant supernatant suspensions, the sediments were resuspended in distilled water and pooled. Up to this point in the separation, the sediment obtained from each centrifuge tube of crude Mickle product had to be processed individually. Also, detergent is necessary until protoplasm and nondispersible aggregates are removed, after which distilled water must be used for a suspending medium. The sediment obtained from centrifuging the aqueous pool consisted of a translucent upper layer of cell walls and an opaque lower layer of unbroken cells. Repeated resuspensions and centrifugations of the upper layer in water, until only one layer was discernible, resulted in complete separation of cell walls from intact cells. Electron micrographs revealed that cell walls of mycobacteria obtained by the Mickle process were not fragmented, but were merely cracked at one end of the rod and that these cell-wall preparations contained little or no protoplasmic remnants (Fig. 1).

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* We recently observed that a commercial pressure cell can be utilized in place of the Mickle apparatus to increase the yield of cell-wall material.

As in earlier studies with *Bacterium tularense*(9), fractions were tested for biological activity by intradermal injection of rabbits. Firm, raised, smooth nodules were produced with preparations of mycobacteria grown on either solid or liquid media and killed by heat, formaldehyde, or ether.

Results. Rabbits were injected intradermally with preparations obtained from strain H37Ra. Preparation No. 1 consisted of whole organisms (1 mg/ml) in Tween solution containing 0.4% formalin, No. 2 of whole organisms (1 mg/ml) in Tween solution saturated with ether, No. 3 of protoplasm (3 mg/ml) in 0.2% formalin, and No. 4 of cell walls (0.2 mg/ml) in 0.4% formalin. Each of 2 rabbits received 0.2-ml amounts of a series of 2-fold dilutions in 0.85% NaCl solution from undiluted to 1:128 of each of the 4 preparations. Lesions were produced with whole organisms and cell walls but not with protoplasm. In the first rabbit, 1.6 μg of whole organisms (Nos. 1 and 2) and 0.3 μg of cell walls (No. 4) produced lesions. In the second, similar amounts of whole organisms (Nos. 1 and 2) and 1.25 μg of cell walls (No. 4) produced reactions.

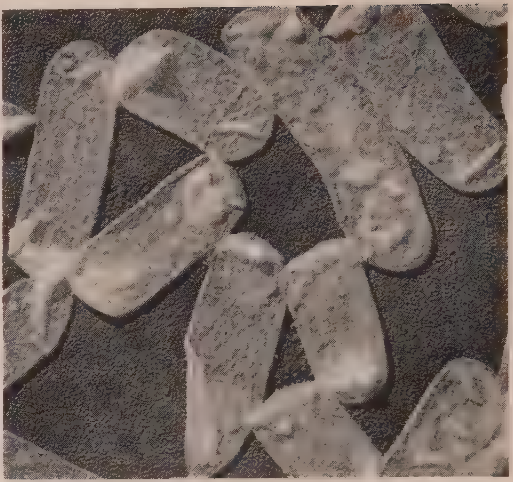

FIG. 1. *Mycobacterium butyricum,* cell-wall preparation, × 25,000.

In another experiment similar results were obtained with BCG. Each of 3 rabbits was injected at different sites with 0.1-ml amounts of serial dilutions of suspensions containing cell walls and of suspensions containing protoplasm in initial concentrations of 0.2 mg/ml and 1.40 mg/ml, respectively. In each animal 0.6 μg of cell walls produced lesions, while 140 μg of protoplasm did not.

Protoplasm and cell walls of *M. butyricum* also were tested. Protoplasm was given in 0.1-ml quantities of serial twofold dilutions containing from 140 μg to 2.2 μg and cell walls in dilutions containing 40 μg to 0.6 μg. Three animals injected only with protoplasm failed to react, and 2 animals given only cell walls developed lesions at sites of injection with 1.25 μg or 2.5 μg of material. Four other rabbits received protoplasm and cell walls at different sites. In none of them did protoplasm produce an effect, whereas each animal reacted to a dose of 1.25 μg of cell walls.

After 22 days, the 6 rabbits which had received cell walls of *M. butyricum*, the 3 rabbits which received an initial injection of protoplasm of *M. butyricum*, and 3 normal animals were injected into separate areas of the skin with 40 μg of cell walls and 40 μg of protoplasm of homologous organisms. At 48 hours postinjection, protoplasm had caused little reaction at the site of inoculation, whereas cell walls had produced severe reaction in the 6 animals that had received an initial injection of cell walls. The lesions were red and edematous and varied from 20 x 7 to 38 x 35 mm in diameter. The 3 animals that had received protoplasm as the initial inoculum developed flat lesions varying from 7 x 7 to 13 x 13 mm in diameter following the second inoculation of cell walls, although the 3 normal animals failed to respond. These results demonstrate that cell walls contain fractions capable of inducing hypersensitivity of the delayed type and of eliciting skin reactions in sensitized animals.

Initial lesions produced by mycobacteria injected into the skin of rabbits do not appear until after 4 or 5 days. In this respect these organisms differ from *B. tularense* and *Hemophilus pertussis* suspensions which produce visible effects in rabbits within 24 hours(10). Initial and sensitivity reactions produced by fractions of mycobacteria can be differentiated by their time of appearance. Details of these and other studies will be discussed later.

Summary. A technic was described for separation of cell wall and internal protoplasm from cells of *Mycobacteria*. Data are presented which demonstrated that cell walls produced lesions when injected intradermally into rabbits, whereas protoplasm failed to produce these lesions. Cell walls were also shown to be capable of inducing hypersensitivity of the delayed type. Separation of these morphological elements resulted in definite separation of one element possessing certain biological activities from material not possessing these characteristics. Such initial fractionation of the cell should facilitate purification and characterization of substances which elicit typical tissue responses to infection with tubercle bacilli.

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Serological Studies on Hemolytic Antigen from Leptospira. (24012)

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A hemolytic (HL) procedure(1), which was a modification of the hemagglutination test of Chang and McComb(2), was reported from this laboratory. More recent reports (3,4) describe the preparation of this HL antigen from non-pathogenic *Leptospira biflexa,* its standardization and stabilization, and its evaluation in serodiagnosis of human leptospirosis. This evaluation demonstrated the generic specificity of the HL antigen, as well as its marked sensitivity with rabbit and human antisera, and suggested that currently used microscopic agglutination tests could be advantageously supplanted by the HL procedure in serodiagnosis of human leptospirosis. In view of its potential usefulness, further serological studies on HL antigen were carried out.

Materials and methods. HL antigen was prepared from *L. biflexa* as previously described(3). Antisera were prepared in rabbits by immunizing them intravenously with suspensions of leptospires(3), suspensions of leptospires previously extracted with ethanol or ether, the HL antigen, or HL antigen adsorbed onto rabbit erythrocytes. A total of 10 ml of HL antigen, containing at least 1024 units/ml(3), was administered over 2 weeks. HL antigen was adsorbed onto sheep or rabbit rbc as previously described(3), and approximately 20 ml of 10% sensitized rabbit rbc (exposed to 640 units HL antigen/ml) were given for 2 weeks. Sheep and rabbit rbc were sensitized with HL antigen in like manner for use in adsorbing HL antibodies from sera. Pre-immunization blood samples were obtained and proved to contain no leptospiral antibodies. Antisera were obtained from rabbits during their initial peak titers. Antiserum adsorptions were carried out at room or refrigerator temperatures as multiple adsorptions, with more than sufficient antigen necessary to remove homologous antibodies. Complement-fixation tests were performed according to usual Kolmer procedure(5), using ½ volumes of all reagents, 4 units of hemolysin, 2 full units of complement, and 1% sheep erythrocytes. Precipitin tests were performed as described by Rothstein and Hiatt(6). HL and microscopic agglutination (MA) titrations were performed as previously described (1,3).

Results. Preliminary studies were made on the chemical nature of HL antigen. Negative ninhydrin and positive Biuret and Molisch tests were obtained on freeze-dried antigen. No significant alteration in titer was obtained by twice deproteinization by the Sevag method, nor by extraction of dried antigen with ether, acetone or chloroform for 2 hours at room temperature. In 24 hours at 37°C and pH 7.4, a final concentration of Bacto-trypsin of 0.1 mg/ml of a solution of antigen containing approximately 2 mg/ml did not alter the titer. Higher concentrations of trypsin, even after inactivation of the enzyme by heat, gave inconclusive results presumably because of its adsorption to erythrocytes. In 4 hours at 37°C 2.5 M urea reduced the titer to ½, and 5 M urea reduced it to ¼ its original activity. Extraction with N/5 HCl in boiling water bath for 10 minutes reduced the titer to 1/16, and similar treatment with N/5 NaOH reduced the titer to 1/64 of its original activity. Additional studies supported Chang's earlier reports that his ESS antigen was stable to temperatures of 100°C for 15 minutes, was not precipitated by trichloracetic acid, was soluble in 50-66% ethanol, relatively soluble in ethanol concentration of 80%, and insoluble in 90% ethanol. Chang(2) suggested that his antigen was "nonprotein," which is supported by above data; however, the positive Biuret and low order of inactivation by urea leads one to conclude that the polysaccharide is probably complexed with some low molecular weight nitrogenous material.

TABLE I. Homologous Antibody Response in Rabbits.

Antisera *vs*	Titers (reciprocal) MA	HL
L. biflexa	10,000	10,000
HL antigen	100	1,000
HL antigen sens. rabbit RBC	10	1,000
L. biflexa ext. with 50% ethanol	10,000	—
75% "	10,000	10
50% ether	10,000	1,000

Homologous antibody responses in rabbits to various antigenic preparations from *L. biflexa* are shown in Table I. As anticipated, whole cells of *L. biflexa* stimulated formation of both MA and HL antibodies. HL antigen was also antigenic when injected by itself or adsorbed onto rabbit erythrocytes. Furthermore, it stimulated formation of homologous HL antibodies, and in addition some agglutinating antibodies. Further evidence was obtained for solubility of HL antigen in 50% ethanol, because 3 extractions of *L. biflexa* with 50% ethanol(1,3) rendered cells incapable of stimulating formation of HL antibodies. Similar extraction of *L. biflexa* with 75% ethanol did not completely remove the HL antigen, nor did extraction with 50% ether.

Tables II and III depict results of adsorption of antisera against *L. biflexa* and rabbit erythrocytes sensitized with HL antigen, with homologous and heterologous antigens. In

TABLE II. Adsorption Studies on *L. biflexa* Antiserum.

Adsorbed with antigen	Titers (reciprocals)		
	MA		HL
	L. biflexa	*L. canicola*	
—	10,000	—	10,000
L. biflexa	—	—	—
L. canicola	10,000	—	—
L. biflexa ext. with 50% ethanol	—	—	10,000
L. biflexa ext. with 75% ethanol	—	—	—
HL sens. sheep RBC	10,000	—	—

Table II *L. biflexa* removed both type-specific (agglutinating) and genus-specific (HL) antibodies, whereas *L. canicola* removed only the HL antibodies. *L. biflexa* cells extracted with 50% ethanol, which previously was shown to extract the HL antigen (Table I), removed only type-specific antibodies; whereas *L. biflexa* extracted with 75% ethanol retained sufficient HL antigen to remove both types of antibodies. It is interesting to note that sheep cells adsorbed with HL antigen removed only the genus-specific homologous antibodies, which would support previous findings (Table I) that very minimal amounts of type-specific antigen adsorbed onto sheep or rabbit erythrocytes. Similar results were obtained by analogous adsorptions of *L. canicola* antiserum, which was included in these studies because of the heterologous relationships between *L. biflexa* and *L. canicola*. In Tables I and III it may be seen that the HL antigen not only contained some type-specific antigen, but that sufficient amounts of it remained adsorbed to rabbit erythrocytes after 3 washings to induce minimal, type-specific, agglutinating antibody response. Adsorption with *L. biflexa* removed both types of antibodies, whereas adsorption with *L. canicola* removed only the HL antibodies. Adsorption with sheep erythrocytes sensitized with homologous HL antigen removed both antibodies (Table III). Adsorption of HL antiserum with *L. biflexa* extracted with 50% ethanol removed the type-specific antibodies as expected, but also reduced the HL titer, which was not expected since *L. biflexa* extracted with 50% ethanol had previously been shown (Table I) incapable of stimulating formation of HL antibodies. These 2 experiments were carried out with separate batches of extracted leptospiral cells, and it seems probable in view of later duplicate experiments that HL antigen represented in this Table had not been completely extracted from the latter batch of cells. Later studies indicated that a fourth extraction in 50% ethanol carried out in a 56°C waterbath for 2 hours may increase the efficiency of extracting HL antigen.

TABLE III. Adsorption Studies on Antiserum against Rabbit RBC Sensitized with HL Antigen.

Adsorbed with antigen	Titers (reciprocals)		
	MA		HL
	L. biflexa	*L. canicola*	
—	10	—	1000
L. biflexa	—	—	—
L. canicola	10	—	—
HL sens. sheep RBC	—	—	—
L. biflexa ext. with 50% ethanol	—	—	100
L. biflexa ext. with 75% ethanol	—	—	—

It was apparent that, by suitable adsorptive procedures, antisera could be prepared which contained only MA or HL antibodies. This permitted studies on precipitating and complement-fixing properties of each of the 2 types of antibodies. Results of precipitin tests using HL antigen and various antisera, adsorbed and unadsorbed, are shown in Table IV. HL antigen gave precipitation in low dilutions only with antisera containing MA antibodies, and this was apparently possible because of MA antigen impurities previously shown to exist in HL antigen. These findings suggest that precipitation was not due to the HL antigen-antibody reaction, which was substantiated by the very low titer obtained with antiserum against HL antigen and the finding that precipitating activity was adsorbed from such serum with 50% ethanol extracted *L. biflexa*.

L. biflexa cells, 50% ethanol extracted *L. biflexa*, and HL antigen were used as antigens in complement-fixation tests on homologous and heterologous rabbit antisera (Table V). All 3 antigens fixed complement only with antisera containing type-specific, MA anti-

TABLE IV. Precipitin Tests.

Rabbit antisera								
		Titer (reciprocals)		HL antigen dilutions				
vs	Adsorbed with	HL	MA	Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}
L. biflexa cells		1,000	10,000	+*	+	±	—	—
L. biflexa ext. with 50% ethanol	Rabbit RBC ads. with HL antigen	<10	"	+	+	—	—	—
HL antigen		1,000	100	+	—	—	—	—
Rabbit RBC ads. with HL antigen	*L. biflexa* ext. with 50% ethanol	10,000	<10	—†	—	—	—	—
Non-immunized		<10	"	—	—	—	—	—

* Positive results indicated were obtained only with antiserum dilutions of 1:4 or lower.

† Negative results obtained with undiluted antiserum, and double dilutions through 1:1024.

bodies. That complement-fixation with HL antigen was due to MA antigen impurities was shown by finding that antiserum with a high titer of only HL antibodies showed no complement-fixation with any of the 3 antigens. The possibility was considered that complete lysis (no fixation) might not be due to lack of complement-fixation, but rather to the HL reaction itself. Accordingly, appropriate controls lacking only hemolysin were included in this series of complement-fixation tests. Absence of hemolysis in these controls indicated that lack of complement-fixation in the indicated tests was actually due to absence of complement-fixation between HL antibodies and their homologous antigen in the impure HL antigen preparation.

Discussion. Apparently the HL antigen-antibody system is not operative in leptospiral agglutination, precipitating, or complement-fixing reactions. However, the HL antigen is undoubtedly similar, if not identical, to the hemagglutinating antigen (ESS) described by Chang and McComb(2). Complement-fixing antigens described in the past include a deproteinized preparation from lysed leptospiral cells(7), supernatants of whole cultures(8), ethylene glycol extracts of leptospirae(9), sonically disrupted leptospirae(10) and suspensions of whole leptospiral cells(11). Relationships between active antigens in each of these preparations and their serotype specificity remains obscure. A more recent article by Rothstein and Hiatt described a clear precipitinogen obtained by electrodialysis of 70% ethanol extracts of leptospirae. They suggested that this precipitinogen probably contained a mixture of peripheral, type-specific antigen(P) and somatic, genus-specific, lipopolysaccharide antigen (S). They also suggested that the ESS antigen of Chang(2), which is probably very similar or identical to the HL antigen, probably consisted mainly of their S antigen. Although their precipitinogen and the HL antigen both contain polysaccharide, are heat stable and resistant to trypsin, there are several areas of divergence. The precipitinogen possessed complement-fixing

TABLE V. Complement-Fixation.

Rabbit antisera				Reciprocal of CF titers *vs* antigens*		
		Titer (reciprocals)				
vs	Adsorbed with	HL	MA	*L. biflexa* cells	*L. biflexa* ext. with 50% ethanol	HL antigen
L. biflexa cells		1,000	10,000	>128	>128	>128
L. biflexa ext. with 50% ethanol	Rabbit RBC ads. with HL antigen	<10	"	"	"	"
Rabbit RBC ads. with HL antigen	*L. biflexa* ext. with 50% ethanol	10,000	<10	—†	—	—

* All antigens used in dilutions of 1:8 to 1:32, based upon optimal activity determined by block titrations.

† No fixation with undiluted serum, nor with double serum dilutions through 1:128.

activity, and failed to sensitize human erythrocytes to hemagglutination. Furthermore, HL antibodies were easily removed by adsorption with leptospiral cells containing HL antigen, which was contrary to the findings of Rothstein and Hiatt with their precipitinogen. It is possible that variance in adsorptive technics between different laboratories may account for such differences. In this work leptospirae used in adsorptions were freshly harvested, non-formalinized cells. The most striking divergence is the difference in precipitating abilities. The relationship of HL antigen to the antigens of Rothstein and Hiatt remains unclear, although the HL antigen shows more resemblance to S than to P antigen. It is probable that the HL antigen and ESS, as suggested by Rothstein and Hiatt(6), contain their S antigen, and that differences in characteristics reflect differences in methods of preparation. Further clarification of relationships between leptospiral antigens and their chemical natures must await investigations using purified preparations, and purification will be difficult until leptospirae can be cultivated in chemically-defined, non-protein media.

Summary. Use of HL antigen adsorbed onto erythrocytes, and *L. biflexa* repeatedly extracted with 50% ethanol, permits preparation of antisera containing only type-specific (agglutinating) or genus-specific (HL) antibodies. HL antigen, as extracted from *L. biflexa* with 50% ethanol, contained small quantities of type-specific antigen, which was capable of adsorbing onto rabbit and sheep erythrocytes along with the HL reactive antigen. However, the type-specific antigen was apparently not operative in the hemolytic reaction. HL antibodies would not precipitate HL reactive antigen, agglutinate leptospiral cells, nor fix complement in the presence of HL reactive antigen or *L. biflexa* cells. Preliminary studies indicate that HL antigen contains polysaccharide which is probably complexed with some low molecular weight nitrogenous material.

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Inhibition of Arthus Reaction During Protracted Systemic Anaphylaxis in Mice and Guinea Pigs.* (24013)

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During studies on anaphylaxis in mice sensitized with antigens incorporated in paraffin oil adjuvants, it was found that protracted anaphylactic shock elicited by intralabial injections of antigen inhibited Arthus reactions at sites of injection. Furthermore, when mice sensitized to both hemocyanin and human serum albumin were injected into the lip with hemocyanin and intravenously with human serum albumin, Arthus reactions were scant or failed to occur. Similarly sensitized mice given only intralabial injections developed intense local reactions(1). Our observations were in accord with work of Bier *et al.*(2) who found that systemic antigen-antibody reactions in the rat alter its reactivity in passive cutaneous anaphylaxis (PCA) tests involving other antigen-antibody systems. Inhibition of PCA reactions after (intraperitoneal) injection of a nonrelated antigen and (intravenous) injection of its corresponding antibody was compatible with the hypothesis that complement (C′) or components of complement have a contributory role in the local reaction. Rats were chosen for these experiments because of low normal C′ levels permitting decomplementation by means which might prove lethal in the guinea pigs, and because of the purported refractoriness of rats to systemic anaphylactic shock. Further studies by Osler *et al.*(3) indicated that the amount of third component (C′3) of C′ present in the circulation is critical insofar as PCA reactions are concerned, but participation of some or all other components in the local reaction was not ruled out and was considered a probability. We have found that a severe protracted anaphylactic shock can be consistently produced in sensitized guinea pigs after subcutaneous eliciting injections of antigen(4). This observation made it possible to study systemic antigen-antibody reactions on local hypersensitivity in this species (which has relatively large amounts of circulating C′3).

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In experiments to be described, mice and guinea pigs were sensitized with 2 unrelated antigens, and the effect of systemic anaphylactic shock upon Arthus reactions was observed. As in the mouse, anaphylactic shock in the guinea pig was accompanied by a decrease of Arthus sensitivity or reactivity.[†]

Methods and materials. Adult male Hartley guinea pigs and adult male albino mice were used. Guinea pigs were sensitized by single subcutaneous or intracutaneous injections of 2.25-2.5 mg of egg albumin (Ea) or by both this amount of Ea and 0.025-0.05 ml of washed packed sheep erythrocytes (SRBC) incorporated together in paraffin oil adjuvant without mycobacteria. One group of mice was injected with both 1 mg of human serum albumin (HuSA) and 0.08 mg N of Busycon hemocyanin (BH) emulsified together in complete adjuvants containing 0.2 mg of *M. tuberculosis*. Other mice were sensitized with both chicken egg albumin (Ea, 0.5 mg) and the BH incorporated into one dose with complete adjuvants. For details of preparation of antigens in paraffin oil adjuvants see(6). For testing local sensitivity in mice, a 30 gauge needle was used for injections into the lip(7) or footpad(8). The volume of fluid injected was 0.05 ml in the lip and 0.03 ml in the footpad. Intralabial injections were made into the dry mucous membrane of the free edge of the upper lip, the needle entering parallel with the skin. Reading of labial reactions were made as follows: 1+ = swelling noted by comparison with uninjected lip; 2+ = swelling obvious without comparison; 3+ = swelling of lip, cheek, and upper "snout"; 4+ = resembles profile of moosehead. (For details on intraperitoneal immunization and intralabial testing of mice, see 7). Rectal temperatures of mice and guinea pigs were recorded before and after eliciting injections

TABLE I. Arthus Reaction and Systemic Anaphylaxis after Intralabial Injection of Sensitized Mice.

Antigen*	Days after last inj.	Anaphylactic death[†]	Labial Arthus[‡]
Horse serum	7– 8	1/30	15/29
Human serum albumin	9–13	10/20	3/11
Hemocyanin	7–16	1/10	8/ 9
Horse serum globulin	13	0/ 6	6/ 6
Human serum globulin	18	0/15	15/15
Egg albumin	37	1/ 4	3/ 3

* Sensitization consisted of 2 intraper. injections of antigen 3 wk apart. Mice were given eliciting injections of homologous antigen (0.05 ml of HS, 3-5 mg of HuSA, 0.8 mg N of BH, 8-9 mg of HGG, 8.25 mg of HuGG and 0.5 mg of Ea).

[†] Numerator is No. of mice dead of anaphylactic shock within 24 hr; denominator is No. of mice challenged.

[‡] Reading of 2+ or greater at 4 hr after eliciting injection.

[†] Described in part in abstract: *Excerpta Medica*, July 1958(5).

TABLE II. Inhibition of Arthus Reactions in Mice Sensitized to 2 Unrelated Antigens.

Sensitizing antigens* Mice sensitized to Busycon hemocyanin and	Route of challenge† Lip	I.V.	Anaphylactic death‡ 1 hr	4 hr	24 hr	Anaphylactic symptoms§ 1 hr	Arthus‖ 4 hr
Human serum albumin	BH	HuSA	1/ 9	2/ 9	2/ 9	5/ 8	1/ 7
Idem	"	None	0/ 5	0/ 5	0/ 5	0/ 5	5/ 5
Egg albumin	"	Ea (.1 mg)	2/ 5	2/ 5	2/ 5	1/ 3	0/ 3
Idem	"	Ea (.05 ")	3/10	3/10	3/10	6/ 7	0/ 7
"		None	0/10	0/10	0/10	2/10	7/10

* Both antigens were incorporated in complete adjuvants containing 0.2 mg *M. tuberculosis* and inj. by intraper. route. Mice were challenged 16 days after a second inj. (HuSA-BH) or 21-22 days after a single inj. (Ea-BH).

† Intrav. inj. immediately preceded intralabial inj. of .08 mg N of BH.

‡ Numerator is No. of deaths until hour listed; denominator is total No. of mice challenged. This is accumulative; thus, the figure in 24 hr column is total of mice which died in 24 hr period.

§ Dyspnea and/or bristling of fur and/or collapse.

‖ Reaction of 2+ or greater.

TABLE III. Anaphylactic Syndrome and Arthus Reaction in 16 Mice Sensitized to Busycon Hemocyanin and Egg Albumin and Injected Intralabially with Hemocyanin.

Temp. before inj., °C	Amt of Ea inj. I.V.,* mg	Reactions, 1 hr Anaph.†	Temp., °C	Arthus‡	Reactions, 4 hr Anaph.†	Temp., °C	Arthus‡
38.8	.3	BF, D	32.9	0	C	34.4	0
37.3	.1	N	37.5	0	N	38.1	0
37.9	.1	BF, D	31.9	0	BF, D	34.6	0
37.5	.1	N	36.5	0	N	37.5	0
38.3	.05	Hy	35.2	±	N	37.1	0
38.3	.05	BF, D	32.5	±	BF, D	33.9	0
38.4	.05	D	34.8	1	N	38.0	1
38.5	.05	N	37.5	2	N	37.7	1
38.6	.05	BF, D, C	32.9	0	N	37.5	0
38.0	.05	BF, WG	36.3	±	N	37.8	1
39.0	.05	WG	35.5	1	N	37.9	0
38.3	.0	N	38.8	1	N	38.4	3
38.5	.0	N	38.7	2	N	39.5	3
38.9	.0	N	39.1	3	N	39.1	2
37.9	.0	N	37.8	1	N	38.6	1
38.0	.0	N	38.2	3	N	37.7	2

* Eliciting injections 21-22 days after sensitization. Hemocyanin inj.: .08 mg N.

† BF, bristling of fur; D, dyspnea; Hy, hyperexcited; WG, wobbly gait; C, collapse; N, apparently normal.

‡ Reactions ± to 4+ described in methods.

of antigens. TRI-R‡ electronic thermometers were used with appropriate probes for the 2 species of animals.

Results. Experiments in mice: It was observed in preceding studies that mice sensitized with various antigens in paraffin oil adjuvants frequently died of anaphylactic shock after an intralabial injection for testing Arthus sensitivity(7). Mice suffering ultimately lethal or severe anaphylactic shock had scant or no Arthus reactions in the lip. Table I shows some of the results of intralabial injections of homologous antigen into sensitized mice. When the antigen caused a high percentage of severe systemic reactions (HuSA) there were few Arthus reactions of more than 2+ intensity in surviving mice; when the antigen did not induce severe systemic reactions (BH), intense Arthus reactions were demonstrable in a large percentage of mice. Furthermore, mice in readily apparent shock did not react at labial sites of injection.

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‡ Model TML, TRI-R Instrument Co., Long Island City. Probe for rectal temperatures in mice TP-5; for rectal temperatures in guinea pigs, TP-1B.

TABLE IV. Anaphylaxis and Arthus Reaction in 17 Mice Sensitized to Busycon Hemocyanin and Egg Albumin and Injected into Foot Pad with Hemocyanin.

	Reactions, 1 hr			Reactions, 4 hr		
I.V. inj. of Ea*	Anaph.†	Temp., °C	Arthus‡	Anaph.†	Temp., °C	Arthus‡
.05 mg	BF, WG	32.5	0	N	37.8	0
	N	37.3	±	N	38.1	2
	BF, WG	33.9	0	N	38.4	2
	N	35.6	1	N	38.2	1
	D	35.9	0	N	38.6	0
	N	37.7	1	N	38.0	1
	N	38.4	1	N	38.6	1
.0 mg	N	39.2	2	N	38.0	3
	N	38.5	1	N	36.7	2
	N	39.0	3	N	37.7	4
	N	39.5	2	N	37.7	3
	N	39.1	1	N	38.0	3
	Mild BF & D	38.7	2	N	36.1	2
	Idem	36.4	1	N	37.0	3
	N	38.2	1	N	37.5	3
	N	38.7	2	N	37.9	3
	N	39.2	1	N	37.9	4

* Eliciting inj. 1 wk after mice were used for intralabial testing. Mice inj. at that time with BH and Ea were challenged with BH and Ea; mice inj. at that time with BH only were challenged only with BH. BH dose: .03 mg N.

† BF, bristling of fur; D, dyspnea; WG, wobbly gait; N, apparently normal.

‡ Reactions ± to 4+ corresponding to increasing amounts of edema (the reaction in feet is described in reference 8).

We investigated this inhibition in mice sensitized with 2 unrelated antigens incorporated in complete adjuvants. Table II shows that a systemic antigen-antibody reaction can also interfere with local reactions involving another antigen-antibody system. Intravenous injections of Ea or HuSA in mice sensitized to these antigens and hemocyanin have an inhibitory effect on the labial Arthus reaction to hemocyanin. The systemic antigen-antibody reaction under these conditions elicited a typical shock syndrome. The mice in shock had dyspnea, bristling of fur, and a fall in rectal temperature. Table III shows the relationship of shock symptoms to labial Arthus reactions in these mice. Degree of shock and degree of inhibition of local reactions were apparently related. One week after the intralabial tests, similar experiments were undertaken using the same mice, which were this time tested in the footpad with BH. Similar results were observed, *i.e.* local reactions were scant or did not occur in mice showing shock symptoms due to the systemic reaction to Ea (Table IV).

Experiments in guinea pigs. Table V shows results of subcutaneous injection of varying amounts of Ea into guinea pigs sensitized to Ea 25-32 days previously. The guinea pigs which received doses of antigen capable of eliciting severe protracted anaphylactic shock did not have Arthus reactions at sites of subcutaneous injection. Five of 7 guinea pigs injected with 1 to 5 mg of Ea had distinct local reactions. No guinea pigs injected with this amount of Ea subcutaneously showed any signs of anaphylactic shock.

Table VI shows that the results in guinea pigs sensitized to 2 antigens are like those in the mouse. At 4 hours there was complete inhibition of the Arthus reaction to Ea in guinea pigs showing shock symptoms. Twenty-four hours after the challenging injections of SRBC, there were reactions in the skin of guinea pigs which had suffered the anaphylactic shock. However, these differed grossly from reactions in guinea pigs not injected with sheep erythrocytes. The late reactions were characterized by a uniform purple color; there was no noticeable edema. The 24-hour responses in control animals were typical Arthus reactions; *i.e.* there was redness and swelling, with signs of necrosis in the center of the lesions.

Discussion. The mechanism of inhibition of local anaphylactic reactions by a systemic antigen-antibody reaction cannot be elucidated on the basis of evidence thus far available. Our experimental results are in harmony with at least 2 explanations which are not mutually exclusive:

(1) Local anaphylactic reactions depend on at least 3 factors, antigen, antibody, and complement or a component or components of complement. Complement removed by a systemic antigen-antibody reaction may not be available for a contributory role in the local reaction. This is the hypothesis of Bier and Osler(2,3) with regard to inhibition of passive cutaneous anaphylaxis in the rat. They emphasized, however, that their recent studies

TABLE V. Arthus Reactions and Systemic Anaphylaxis after Subcutaneous Injection of Sensitized Guinea Pigs.*

Amt of antigen in chall. dose	Anaphylactic reactions‡		Arthus reactions§	
	3-7 hr	24 hr	3-7 hr	24 hr
100 mg	† (3½)		0	
	† (4)		i.d.h.	
	Sev.	†	0	i.d.h.
	† (3)		±	
	Sev.	Sev.	0	0
50	Sev.	0	0	0
	"	Mild	0	0
	"	†	0	† NR
	† (1½)			
	Sev.	†	0	† NR
	"	†	0	0
20	Mild	0	0	0
	"	0	P10×10	RS40×45×2
	Mod.	Mod.	0	0
5	0	0	0	0
	0	0	PS40×40×SS	RS50×50×SS
	0	0	NeS15×15×SS	PS40×50×SS
	0	0	R10×10	PS40×50×SS
	0	0	0	0
1	0	0	P10×10	P10×10
	0	0	P10×10	0

* Guinea pigs were sensitized by single subcut. inj. of 2.5 mg of egg albumin in incomplete adjuvant.

‡ Sev. = severe; Mod. = moderate; 0 = no anaphylactic symptoms; † = death. Figure in parentheses denotes hour of death.

§ i.d.h. = ill defined hemorrhagic reaction; ± = doubtful reaction; P = pinkness; R = redness; Ne = necrosis; S = swelling; SS = slight swelling; NR = not read; 0 = no reaction. Dimensions of lesions are in mm; thus 40×45×2 = reaction 40 mm by 45 mm, raised 2 mm.

did not provide unequivocal proof as to the absolute requirement for C′ in initiation of PCA(2). Serum C′ may merely serve to potentiate the PCA reaction by "intensifying" the interaction of antigen with antibody.

(2) Systemic antigen-antibody reactions cause a series of physiological changes which include alteration of capillary permeability and fall in blood pressure. There may be interference with antigen-antibody union at the local site and/or inhibition of results of that union, *i.e.* interference with participation of cellular or humoral factors from the circulation.

Osler *et al.*, pointing out that analogies made between *in vitro* and *in vivo* systems are subject to criticism, look at PCA as the final step in a series of events corresponding to an *in vivo* C′ fixation reaction in the presence of a large excess of C′ supplied by the circulating blood(3). However, anaphylactic type reactions differ in several respects. It should be noted that Mongar and Schild find that C′ is not a factor in anaphylactic contraction of smooth muscle or in release of histamine(9). The Arthus reaction is more complex and more lasting than PCA, involving cellular elements from blood. Complement may be less important in the Arthus reaction than in PCA. There are several points which argue against the concept of C′ having a major role in eliciting Arthus reactions. Mice sensitized with various antigens in complete adjuvants show strong Arthus reactions after intralabial injection of homologous antigen, despite the low level of circulating complement in mice (1 to 2 $C'H_{50}$ units/ml, personal communication, J. A. Bauer) limited by very low titers of C′2 and C′3(10). Guinea pigs have large amounts of circulating C′ and are difficult to decomplement by injection of antigen-antibody combinations or zymosan (personal communication,

TABLE VI. Inhibition of Arthus Reactions to Egg Albumin in Guinea Pigs Sensitized to Both Sheep Erythrocytes and Egg Albumin. Subcutaneous injection of sheep erythrocytes and intracutaneous injection of egg albumin.

Guinea pig	Subcut. inj. SRBC	Anaphylactic reactions*			Arthus reactions‡		
		2 hr	4 hr	24 hr	2 hr	4 hr	24 hr
§	0.25 ml of 10% susp.	†(2½)			P 10×10		
§		Not read	BF,D↓	†(25)	Not read	0	Pl 15×18
§		BF,D	”	R	0	0	Pl 20×15
§		”	”	R	0	0	Pl 10×10
‖		”	BF,D	R	0	0	Pl 15×18
‖		”	D	R	0	0	0
‖		”	†(4)		0		
§	None	0	0	0	RS15×15×2	PS18×18×SS	RS30×30×SS
§		0	0	0	RS20×20×2	PS25×20×SS	P25×25
§		0	0	0	R15×18×3	PS15×15×SS	PS40×40×SS
§		0	0	0	PS10×10×3	PS25×20×3	RS35×35×SS
‖		0	0	0	PS12×12×SS	RS20×20×2	RS 8× 8×SS
‖		0	0	0	PS25×25×5	PS30×30×3	RS40×40×SS
‖		0	0	0	PS25×25×5	PS20×25×2	P30×30

* Anaphylactic reactions—BF = bristly fur; D = dyspnea, † = dead (figure in parentheses denotes hour of death); ↓ = hypothermia (temperature 37° or below); R = apparent recovery; 0 = apparently unaffected.

‡ Arthus reactions—Guinea pigs were inj. intradermally with 0.1 ml of 1% crystalline egg albumin. Dimensions are in mm; thus 25×25×2 denotes a reaction 25 mm long by 25 mm wide, raised 2 mm. Pl = purple; R = redness; P = pinkness; S = swelling; SS = slight swelling; 0 = no reaction.

§ Sensitized by 1 inj. of egg albumin and sheep erythrocytes in incomplete adjuvant given intradermally.

‖ Sensitized by 1 inj. of egg albumin and sheep erythrocytes in incomplete adjuvant given subcut.

J. A. Bauer). However, inhibition of Arthus reactions in our guinea pigs during anaphylactic shock would require complete or almost complete decomplementation if C′ were a critical factor. Experiments concerned with the mechanism of inhibition of the Arthus reaction are in progress.

Summary. Arthus reactivity was diminished or abolished in mice and guinea pigs showing protracted anaphylactic shock. Mice were sensitized to 2 unrelated antigens, then injected with one of these antigens into the lip or footpad and with the other antigen intravenously. The reaction in the lip or footpad was scant or did not occur. Guinea pigs in protracted anaphylactic shock after subcutaneous injection of antigen did not react locally 4 hours after skin tests with an unrelated antigen to which they were also sensitive. Reactions were observable 24 hours after intracutaneous injections; these differed grossly from those found in control guinea pigs not injected with subcutaneous shocking doses of antigen.

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Nuclear Size Changes During Autolysis in Normal Mouse Liver, Kidney, and Adrenal Gland.* (24014)

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The term, autolysis, was first used by Jacoby(1) to describe the process of cellular self-digestion. Since then, many workers studied the phenomenon from the standpoint of morphological and chemical changes. In recent years, a cytochemical study(2) and some biochemical studies(3,4) were reported dealing with aspects of this process. The present paper is a continuation in a series dealing with nuclear size changes in mouse tissues(5, 6) and reports the results on autolyzing mouse liver, kidney, and adrenal gland.

Materials and methods. Nuclear size measurements were obtained from adult mouse tissues. Two to 3 mm of tissue were cut from liver and renal cortex and removed along with whole adrenal glands. They were placed in a dish and kept moist with 0.9% saline in 37°C oven for varying periods of time. The dish was kept partly covered to minimize bacterial contamination. Subsequent staining showed that this potential complication was of no importance here. Tissues were removed at definite times and were fixed in 50% formalin. Control samples of tissues from the same animal (or litter in the case of adrenal gland) were fixed immediately upon removal from the animal(s). Paraffin sections were made and all tissues to be compared were mounted on the same slide and stained by Feulgen technic(7). Nuclei were observed under oil immersion and their sizes were measured from camera lucida drawings. The usual precaution was taken to standardize all drawings by keeping the camera lucida at a 45° angle. Measurements of nuclear diameters were made to nearest 0.1 cm (0.1 cm = 0.37 μ). In some cases, slightly aspherical nuclei were measured. Here, the major and minor axes were averaged to obtain mean diameter. Nuclear volumes were computed by considering the round or slightly aspherical nuclei to be spheres and letting their volumes be a function of the diameter cubed. About 200 interphase nuclei were measured in each sample from renal cortex and adrenal cortex (zona fasciculata). About 500 interphase parenchymal nuclei were measured from each liver sample. Standard statistical analyses were made on data from kidney and adrenal gland utilizing the IBM 650 at the Texas A. and M. Computing Center. Because of the complication of polymodal classes in liver statistical analyses were not made. Approximately 6000 nuclei were measured and reported here.

Results. Fig. 1 summarizes nuclear size changes during autolysis in renal and adrenal cortex. After 30 minutes incubation, the average nuclear size in adrenal cortex increased about 70% over the controls. This was followed by gradual decrease to control value after 2 hours' autolysis. In kidney tissue, nuclei increased in size over the control by 70% reaching a peak volume at one hour or soon afterward. This rise was followed by rapid decrease eventually leveling off to a volume of half that of the control in 28 hours. Statis-

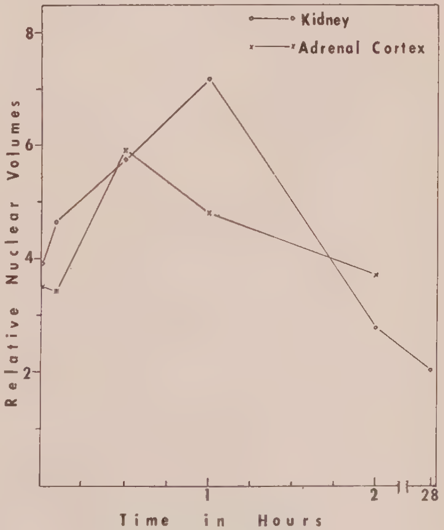

FIG. 1. Changes in avg nuclear sizes in mouse renal cortex and adrenal cortex during autolysis.

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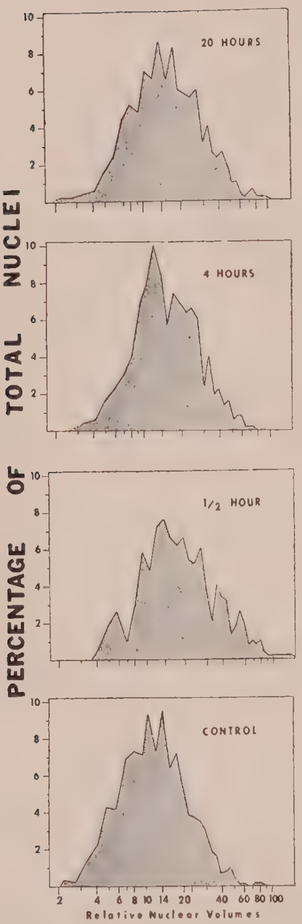

FIG. 2. Frequency polygons of nuclear size distributions from livers of control and autolyzed samples.

tical analyses of data from adrenal cortex revealed significant differences in nuclear size at the 1% level or less, between control tissue and tissue autolyzed for one-half and one hour. In the kidney, significant differences were found between control tissue and those autolyzed for one-half, one, 2, and 28 hours. Some results of autolysis of liver tissue are summarized in Fig. 2. The relative nuclear volumes are compared; no attempt was made to group original measurements into classes. The situation here is complicated by the large spread in nuclear volumes seen in all samples. There may be considerable overlap between different modal groups in each distribution to cause such continuous distributions. Relative nuclear volumes range from about 2 to 100 with the most frequent values at 8 to 20. Any obvious differences between control tissue and autolyzed samples are not apparent in these distributions. Grouping the raw data into classes only serves to reduce the spread and does not reveal anything significant.

Discussion. The term, autolysis, was coined by Jacoby in 1900(1) 10 years after Salkowski(8) showed that softening of dead tissues is brought about by intracellular enzyme action. The early studies on autolysis are summarized by Wells(9), especially from the chemical standpoint. According to Karsner (10), one of the striking changes observed in cells undergoing autolysis occurs in the nucleus. This structure often stains with neutral red or methylene blue, even before morphological alterations appear. Such alterations include paler staining followed sometimes by complete lysis. Lysis of nuclear membranes was not observed in any tissues studied here.

The nuclear size changes reported here in autolyzing kidney and adrenal gland may result from movement of water or other small inorganic molecules through the nuclear membrane. Changes found in autolyzed kidney and adrenal gland might also result from breakdown and displacement of organic constituents. The fact that nuclear volumes could still be measured in Feulgen preparations following extended autolysis indicates that some deoxyribonucleic acid (DNA) was still present. However, cytophotometric measurements would be required to evaluate this problem fully. Leuchtenberger(2) reported a progressive loss of DNA in pycnotic nuclei of autolyzing cells of mouse liver and Sarcoma 180. According to Roslansky and Alfert(11), autolyzing guinea pig kidney slices retain all their DNA after 24 hours.

Degradation of nuclear proteins could also account for nuclear size changes. Alfert(12) recently reported that pycnotic nuclei in autolyzing mouse kidney slices show increased histone stainability. This was also reported (12) in pycnotic nuclei of guinea pig ovary. Leuchtenberger(2) found that about 72-85% of Millon-protein was lost by final stages of pycnosis in mouse liver and Sarcoma 180.

Ordinarily, necrosis refers to changes in cells after death while in the body(13) but autolysis is usually used to describe specific intracellular effects of enzyme action on cells either in or removed from the body. These terms were used somewhat synonymously by Berenbom *et al.*(3,4) in recent studies. These studies dealt with chemical changes associated with mouse liver necrosis *in vivo* and *in vitro*. Ribonucleic acid (RNA) showed the most dramatic change, being reduced to 4% of its initial value 48 hours after pieces of liver were placed in the peritoneal cavity. DNA and protein nitrogen decreased much more gradually than did RNA. However, rate of disappearance of DNA and protein nitrogen was greater *in vitro* than *in vivo*. The fact that initial autolytic changes in kidney and adrenal gland in our study produce larger nuclei and are followed by decrease in nuclear size suggests that more than one cell constituent is undergoing change.

Summary. Small pieces of mouse kidney, liver, and whole adrenal glands were allowed to autolyze in incubator for varying times before fixation. Nuclear sizes were measured and compared with control tissues that had been mounted and stained together on the same slide. Nuclear volumes increased about 70% over controls during the first 30 to 60 minutes of autolysis in kidney and adrenal cortex. This was followed by a decrease in volumes eventually leveling off to about half that of the control in the kidney and the same as the control in adrenal cortex. A comparison of frequency distribution of nuclear volumes in liver revealed no differences between autolyzed samples and the control samples.

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Reaction of Brain Copper Proteins with Sodium Diethyldithiocarbamate in Normal and in Hepatolenticular Degeneration.* (24015)

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A previous report(1) described the separation from brain tissue under copper-free conditions of 3 different copper-containing fractions: fraction I, extracted from fresh tissue with 0.1 M acetate buffer pH 4.5 (or with water or with 0.1 M bicarbonate buffer pH 8.2); fraction II, obtained by subsequent extraction of tissue residue with water at pH 3.5 and vanishing ionic strength; and the residual fraction III. In brains from 2 cases of hepatolenticular degeneration, each with total copper content increased to more than 13-fold the normal, more than ⅔ of the pathological brain copper was extracted in fraction I in a form quantitatively undialyzable at pH

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8.2, presumably bound to proteins. Cerebrocuprein I(2), an essentially homogenous brain protein containing 0.25 to 0.3% copper, has been isolated from bovine fraction I. The copper in cerebrocuprein I was observed to react directly with sodium diethyldithiocarbamate when this reagent was added directly to a solution of copper-containing protein without previous digestion. Since the copper in both ceruloplasmin(3) and hepatocuprein(4), 2 other copper proteins in which this reaction has been studied, fails to react directly, it was considered possible that the direct reaction exhibited by cerebrocuprein I might have been an artifact due to modification of properties of the naturally occurring brain copper protein by the rather rigorous procedures employed during its isolation. Attempts to investigate this possibility by determining the direct-reacting copper in fresh fraction I extracts of bovine brain were unsatisfactory because of the relatively low copper concentration of such extracts in proportion to the abundance of heme compounds also present, and the high absorption of the latter at the wave length employed in measuring the direct reaction. The present paper describes further study of the extent to which the copper in crude bovine cerebrocuprein I prepared by a less drastic method and in fraction I copper proteins from normal human brain and brains from 2 cases of hepatolenticular degeneration reacts directly with sodium diethyldithiocarbamate.

Methods and material. Precautions to avoid contamination with extraneous copper, the general conditions of fractionation procedures, criteria for selection of normal human brains for fractionation, and the methods used for tissue homogenization and for copper analysis were all identical with those employed in previous work(1,2). The direct-reacting copper was determined by a modification of the procedure of Gubler *et al.*(5). Aliquots of a solution of the brain fraction to be analyzed were placed directly in the spectrophotometer cuvette, sufficient redistilled water was added to make the total volume 3.25 ml, 0.5 ml of a saturated solution of sodium pyrophosphate were added, and the optical density (D_1) at 445 mμ measured in Coleman Junior spectrophotometer. 0.25 ml of a 0.1% aqueous solution of sodium diethyldithiocarbamate were added, the solution thoroughly mixed, and the optical density (D_2) measured at 10-minute intervals for the following 60 minutes. By the end of this period, the optical density had in all cases ceased to change significantly. The change in optical density of a reagent blank (D_3) after addition of carbamate was also measured. The net change in optical density 60 minutes after addition of carbamate (Δ OD) was calculated as $D_2 - (D_1 \times 0.938 + D_3)$ and the corresponding quantity of copper calculated from a standard curve. 89 to 110% of copper added to protein samples before pyrophosphate could be recovered as direct-reacting copper. Crude bovine cerebrocuprein I (Cu content 0.15%) was prepared by the procedure previously described(2) as method A (including preparative paper electrophoresis) with the following modifications: (a) acetone precipitates were not dried; (b) dialysis was carried out for only 24 hours, but with constant mechanical shaking; (c) the precipitation at pH 8.2 and the third and fourth acetone precipitations were omitted; (d) the second acetone precipitation was carried out after addition of 1/10 volume of 0.1 M acetate buffer pH 5.2 to the dialyzed solution. The brains of the 2 cases of hepatolenticular degeneration, described previously(1), had been stored at –15° as frozen whole brain (Case A) or as lyophilized dialyzed bicarbonate extract (Case B). The normal human brains had been stored at –15° for 3 months. Copper proteins in pH 4.5 and pH 5.2 extracts from normal human brain were concentrated by precipitation with 3 volumes of acetone and the precipitates dissolved in sufficient 0.2 M ammonium bicarbonate to make a 5 to 10% protein solution. A fraction containing 0.14% copper(6) was obtained from pH 8.2 extracts of normal human brain by dialysis, addition of 1/10 volume of 0.1 M acetate buffer pH 5.2 and collection of the material precipitating between 50% and 75% (v/v) acetone concentration. The pH 8.2 extracts from the 2 cases of hepatolenticular degeneration were analyzed without previous

TABLE I. Direct Reaction of Copper in Fraction I Brain Copper Proteins with Sodium Diethyldithiocarbamate.

Species and preparation	μg Cu in sample analyzed	Extent of direct reaction as Δ O.D.*	as μg Cu	as % of total Cu in sample
Bovine crude cerebrocuprein I	7.25	.013	.47	7
Idem	4.63	.012	.42	9
Normal human Fraction I				
As pH 4.5 extract	7.60	.027	.97	13
Idem	9.12	.028	1.03	11
As pH 5.2 extract	5.88	.013	.47	8
” ” 8.2 ” †	20.00	.018	.65	3
Hepatolenticular degeneration Fraction I				
Case A as pH 8.2 extract	4.86	.039	1.45	30
Idem	10.02	.078	2.90	29
Case B as pH 8.2 extract	11.25	.106	3.92	35
Case B repeated‡	20.12	.242	9.00	45

* Net change in optical density 60 min. after addition of sodium diethyldithiocarbamate.

† Material precipitating between 50% and 75% of acetone concentration after dialysis of pH 8.2 extract and addition of 1/10 vol of 0.1 M acetate buffer pH 5.2.

‡ Repeated on extract stored at −10° for 41 days.

acetone precipitation.

Results. The extent of the direct reaction found in crude bovine cerebrocuprein I, in normal human fraction I obtained by extraction of the tissue at various pH's and in fraction I from 2 cases of hepatolenticular degeneration is shown in Table I. Since the sample of cerebrocuprein I analyzed was only about 50% pure, as indicated both by its copper content of only 0.15% and by ultracentrifugation studies,† it seems probable that the observed direct-reacting copper, amounting to from 7 to 9% of total copper in samples, may represent copper other than that attached to cerebrocuprein I and that the copper in pure cerebrocuprein I does not react directly. The present evidence indicates that the previously observed direct reaction of cerebrocuprein I was an artifact due to the rigorous procedures of isolation and that cerebrocuprein I is, in fact, similar to ceruloplasmin and hepatocuprein in having a copper-protein bond of a type which retains copper against the competing action of sodium diethyl-dithiocarbamate. More than 85% (2.1 to 2.4 μg/g fresh tissue) of the copper in normal human fraction I also appears to be bound in a form which renders it incapable of reacting directly. It is uncertain whether the small amount of direct-reacting copper (<0.35 μg/g fresh tissue) found in this fraction is naturally occurring and of physiological significance as has been shown for the small percentage of plasma copper reacting directly(5).

In brains from 2 cases of hepatolenticular degeneration 29 to 45% of fraction I copper was found to react directly. This observation supports evidence previously presented(1) that a significant portion of the increased brain copper in this disease is bound in a form different from that comprising the bulk of normal human fraction I copper. The direct-reacting copper in fraction I from cases of hepatolenticular degeneration (>7.5 μg/g fresh tissue *i.e.* more than 20-fold the amount of direct-reacting copper in normal human fraction I) may represent copper bound to brain proteins which are normally copper-free. A large part (>17 μg/g fresh tissue in the 2 cases examined) of the pathological copper accumulating in the brain in this disease, however, appears, like the major portion of the copper in normal human brain, to be bound in a form which renders it incapable of reacting directly with sodium diethyldithiocarbamate.

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† Carried out through courtesy of Prof. J. L. Oncley of Harvard Medical School.

Summary. 1) Less than 15% of copper in crude bovine cerebrocuprein I (Cu content 0.15%) and in fraction I copper proteins from normal human brain reacted directly with sodium diethyldithiocarbamate. 2) In brains from 2 cases of hepatolenticular degeneration 29 to 45% of the fraction I copper reacted directly. This direct-reacting copper, amounting to more than 7.5 μg/g fresh tissue *i.e.* more than 20-fold the amount of direct-reacting copper in normal human fraction I, may represent copper bound to brain proteins which are normally copper-free.

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Effect of Tolbutamide on Serum Cholesterol Levels in Hypercholesterolemic Patients.* (24016)

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On the basis of the mild abnormality in glucose tolerance in most patients with idiopathic hypercholesterolemia, the suggestion has been made that these patients have an abnormality of their energy metabolism that resembles diabetes(1). Because of this, a trial of tolbutamide has been carried out to determine what effect, if any, such treatment would have on the elevated serum cholesterol levels. Eight patients were studied. Control periods of one to 24 months were available, since these patients were selected from a larger group being studied because of hypercholesterolemia. Total serum cholesterols ranged between 300 and 500 mg/100 ml. Tolbutamide administration was begun with 3 g the first day, 2 g the second day and 1 g daily thereafter for 2 months. A diet containing approximately 50 g fat/day was constant throughout control and experimental periods. The patients' weight did not fluctuate more than 2 pounds above or below that at beginning of the experimental period. Total and esterified serum cholesterol determinations were made after one week of treatment and at one or 2-week intervals thereafter.

Results. In 5 of the 8 patients a drop in serum cholesterol levels occurred after one week. In one instance this decrease amounted to 100 mg/100 ml of serum and in the others was approximately 50 mg. In 3 patients there was no change from the control period. After the first week there was a slight rise above control values in 5 patients, and no change in the other 3. All patients that showed a decrease after the first week returned to control value or higher by the second or third week and then cholesterol concentration fluctuated at or above control levels until the end of the 2-month period. In no patient was the serum cholesterol lowered below control levels during the second month of tolbutamide therapy.

Conclusions. Tolbutamide administered to hypercholesterolemic patients in doses of 1 g daily did not result in lowering of serum cholesterol concentrations although temporary reductions occurred in 5 of 8 patients during the first week of drug administration.

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* Tolbutamide (Orinase) was supplied by Upjohn Co., Kalamazoo, Mich., through the courtesy of Dr. E. A. Hawk.

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The Cause of Persisting Hypercholesteremia in Rabbit after Cessation of Cholesterol Feeding.* (24017)

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Our comparative study of cholesterol metabolism of the rabbit and rat undertaken(1), suggested that hypercholesteremia developed more readily in the rabbit than in the rat, when both species ingested excess cholesterol because of several observed metabolic differences. First, the rabbit was observed to absorb a larger fraction of its ingested cholesterol than the rat. Secondly, the liver of the rabbit appeared far less capable of removing excess cholesterol from blood than the liver of the rat. This difference in rate of hepatic withdrawal of cholesterol from plasma did not appear, however, to be due to any peculiarity in rabbit plasma but apparently to some comparative defect in the liver itself. For various reasons(1,2,3), we believed that this defect resided in the hepatic reticulo-endothelial system. Another peculiarity of the rabbit fed excess cholesterol is persistent hypercholesteremia long after cessation of cholesterol feeding. The reason for this lag in return of plasma cholesterol to a normal level has not been determined despite its possible importance, as Zilversmit(4) has recently pointed out. It was therefore thought advisable to investigate this phenomenon. The results of our study suggest that the lag is due to prolonged and slow discharge into plasma of the cholesterol previously accumulated in tissues during the cholesterol feeding period.

Methods. A. *Injection of rabbit hypercholesteremic serum.* To determine and compare rate of disappearance of injected rabbit hypercholesteremic serum with that of rat hypercholesteremic serum, 8 normal, young male rabbits (avg wt: 1211 g) were given 10 ml of pooled rabbit hypercholesteremic serum (avg cholesterol: 802 mg/100 ml) obtained from rabbits made hypercholesteremic by ingestion of excess dietary cholesterol. This serum was turbid and on microscopic examination abounded in chylomicra. An additional 10 rabbits (avg wt: 1226 g) were given 10 ml of pooled rat hypercholesteremic serum (avg cholesterol: 750 mg/100 ml) obtained from rats made hypercholesteremic by experimental elevation of plasma bile acid(5). Rat serum was translucent and completely devoid of chylomicra. A sample of blood was obtained from each rabbit of both series before, immediately, 12 and 24 hours after injection. These samples were analyzed for total cholesterol(6).

B. *Acute reduction of plasma cholesterol of hypercholesteremic rabbits.* Fifteen male rabbits (avg wt: 1890 g) were given a diet containing 2% cholesterol and 3% cotton seed oil for 3 months, then they were bled and again given their usual ration of Purina rabbit chow. After 40 days upon the latter diet, these rabbits were anesthetized with ether, and an inlying plastic catheter was inserted into the right external jugular vein of 9 rabbits(7). One week later, each rabbit was given *via* catheter, approximately 20 mg of heparin and then several minutes later, 40 to 50 cc whole blood was removed by syringe and immediately replaced with 40 to 50 cc of whole blood from a normocholesteremic rabbit. This procedure was repeated 4 or 5 times, always employing a new normocholesteremic rabbit for each exchange, to decrease progressively the plasma cholesterol content of the hypercholesteremic rabbit. Blood samples were obtained from hypercholesteremic rabbits before and immediately, 24, 48, 72, and 120 hours after these blood exchanges, and their cholesterol concentration determined. Estimation of the hematocrit in several animals determined before and immediately after the exchange transfusion, revealed that no significant change had oc-

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TABLE I. Rate of Disappearance of Rabbit and Rat Hypercholesteremic Serum in the Rabbit.

No. of rabbits		Avg wt	Plasma cholesterol (mg/100 ml) Before inj.	Immed. after inj.	12 hr after inj.	24 hr after inj.
			A. Rabbits given rabbit hypercholesteremic serum			
8		1211	68	162	106	106
	Range	855–1815	44–97	143–201	68–180	73–155
	S.E. mean		3.5	5.1	4.9	3.7
			B. Rabbits given rat hypercholesteremic serum			
11		1226	59	149	115	107
	Range	840–1482	30–95	130–182	83–137	66–157
	S.E. mean		2.4	4.8	3.6	3.1

curred. The remaining 6 hypercholesteremic rabbits employed as controls, were bled at the same intervals, except for intervals immediately and 120 hours after exchange procedure. A similar exchange was done on 4 normocholesteremic rabbits, their removed blood (40-50 ml) being replaced with hypercholesteremic blood obtained from hypercholesteremic rabbits plasmapheresed above. Here again the *initially* withdrawn blood of at least 2 hypercholesteremic rabbits was given in the first 2 blood replacements effected in the normocholesteremic rabbit. The blood withdrawn from the normocholesteremic rabbits in each exchange was discarded. Blood samples were obtained at similar times as described above.

Results. As Table I illustrates, excess cholesterol contained in serum of the hypercholesteremic rabbit appeared to disappear as rapidly after its injection into the normal rabbit as did the rat hypercholesteremic serum. However as previously pointed out(1), the plasma of either series was not completely cleared of excess cholesterol even at 24 hour period.

Experimental reduction of cholesterol in plasma of hypercholesteremic rabbit was observed (Fig. 1) to be followed within the next few days by a gradual return to much higher plasma cholesterol values, despite the fact that the animal had been and continued to be upon an almost completely cholesterol free diet.

On the other hand, the 4 initially normocholesteremic rabbits made acutely hypercholesteremic by exchange procedure quickly returned to the same level observed in rabbits that had received rat hypercholesteremic serum.

The 6 control hypercholesteremic rabbits continued to lose some of their cholesterol during the experimental period as would be expected.

Discussion. In previous study of cholesterol metabolism of the rabbit(1) it was observed that the cholesterol present in serum of hypercholesteremic rabbit disappeared after its injection into the rat just as rapidly as the cholesterol of rat hypercholesteremic serum. Similarly in the present experiments, the rabbit also exhibited no greater delay in ridding itself of cholesterol present in rabbit hypercholesteremic serum than that in rat hypercholesteremic serum. Thus the profound difference in lipoprotein spectrum of these 2 types of sera(8,9) did not appear to influence

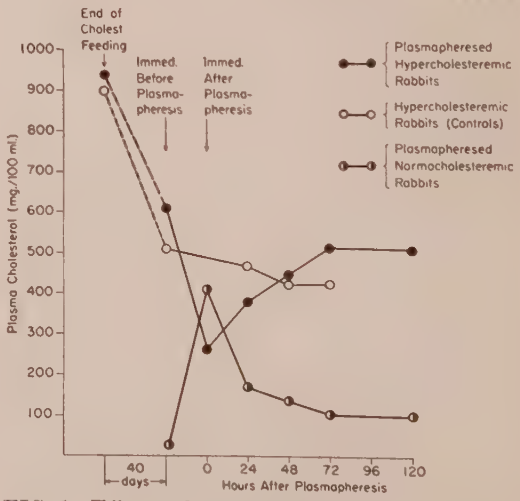

FIG. 1. Effects of acute changes in plasma cholesterol both in hypercholesteremic and normocholesteremic rabbits.

ability of either species to rid themselves of excess cholesterol contained therein. The conclusion therefore appears inescapable that delay in return to normal of cholesterol of the rabbit previously fed excess cholesterol cannot be due to some intrinsic lipoprotein phenomenon in rabbit hypercholesteremic serum which then allows such serum to retain excess cholesterol therein.

The return of plasma cholesterol of hypercholesteremic rabbits to a markedly higher level after its acute reduction by exchange transfusion, of course indicated that such rabbits are receiving cholesterol in their plasma from some tissue(s) either containing an excess of this sterol or else synthesizing and discharging it, at a rate in excess of its rate of hepatic removal and destruction(1). Since the rate of synthesis of cholesterol is not elevated in the cholesterol fed, hypercholesteremic rabbit(1,10), and since an excess of cholesterol remains in various tissues, particularly the liver and aorta(11,12) of rabbits previously fed cholesterol, it would appear that cholesterol rebound after exchange transfusion was due to escape of such tissue excess cholesterol into plasma. Such an escape might continue until some new balance or equilibrium had been established between cholesterol present in tissue and that in plasma perfusing such tissue. It was of interest that normocholesteremic rabbits when made hypercholesteremic by exchange transfusion with hypercholesteremic serum quickly returned almost to their normocholesteremic status, again demonstrating that this type of hypercholesteremic plasma itself possessed no more intrinsic cholesterol, retaining property than that of rat hypercholesteremic serum.

Summary. 1. Normal rabbits were able to rid their plasma of excess cholesterol just as rapidly after such cholesterol was administered in the form of rabbit hypercholesteremic serum, as when administered in the form of rat hypercholesteremic serum. 2. Hypercholesteremic rabbits whose plasma level of cholesterol was reduced by exchange transfusions exhibited a return to higher levels in succeeding days. 3. Normocholesteremic rabbits whose level of cholesterol was elevated by exchange transfusions quickly reverted to their previous normocholesteremic status. 4. These facts suggest that the chronic discharge of previously stored tissue cholesterol is responsible for the long persistent hypercholesteremia in the rabbit after cessation of cholesterol feeding.

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Effect of Homogenizing Medium and Nutritive State on Transaminase Activities of Tissues.* (24018)

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Determination of tissue enzyme activities by use of homogenates requires a medium which will free maximum enzyme activity in a soluble form. Some enzymes show higher activity when the tissue is homogenized in water (1-5) while others are more readily solubilized in isotonic solutions(6,7). A comparison has been made of the effectiveness of water and phosphate buffer in homogenization of tissues for aspartic- and alanine-glutamic transaminase activities in fed and postabsorptive rats and mice.

Methods. Animals: Mice were maintained in glass jars and rats in individual screen bottom metal cages in air-conditioned room at 22-24°C. They were fed *ad libitum* the Jim Dandy ration cubes. The mice were killed by dislocating the vertebrae at base of skull and the rats by blow on head. The animals were bled by cutting blood vessels of the neck. *Enzyme determinations*: Tissues were homogenized in an all glass apparatus at 0°C. Liver and heart were diluted to 1% and the kidney to 2%. Aspartic-glutamic transaminase activity was determined by the method of Cohen and Cammarata(8) with omission of pyridoxal phosphate and pre-incubations. The reactants were prepared in 0.05 M potassium phosphate buffer (pH 7.4) and preheated to 40° in water bath prior to use. Buffer was substituted for the α-keto-glutarate in the blank cell. Formation of oxalacetic acid was continuously measured for at least 5 minutes at 270 mμ with Cary Model 14 spectrophotometer[†] containing a thermostable cell-chamber maintained at 37°C. A comparison was made of the spectrophotometric method with the colorimetric method(9) and a factor derived to convert changes in optical density to units previously used(10). Alanine-glutamic transaminase activity was determined by modification(11) of a colorimetric method for aspartic-glutamic transaminase(9).

Results. Nutritive state: Removal of food on the day prior to autopsy caused a decrease in body and liver weights, a slight decrease in weight of kidneys and no effect on heart. Lack of food did not affect magnitude of transaminase activities in the kidney and heart (Tables I and II). Transaminase activities of liver changed in proportion with change in liver weight, except aspartic-glutamic transaminase in the mouse liver which maintained its total activity.

Buffer vs. water as homogenizing medium: Homogenization of tissues in buffer gave lower values for aspartic-glutamic transaminase activity in liver and kidney but higher values in the heart (Tables I, II). The alanine-glutamic transaminase activity on the other hand was the same in both media for heart and liver but that of kidneys was lower with the buffer preparation. The changes were practically identical for tissues of the rat and mouse in both fed and postabsorption states.

Discussion. The difference in response of the 2 transaminases to homogenization in water and buffer indicates a difference in solubility, tissue combination or denaturation of these enzymes. Furthermore, the degree and nature of the binding varies with the tissues. Both enzymes may be readily mobilized along with other proteins when the exogenous source of foodstuffs is withdrawn. Failure of mouse liver, however, to give up any of its aspartic-glutamic transaminase activity is noteworthy. Apparently, this enzyme is not spared as readily as the alanine-glutamic transaminase. Removal of food has been demonstrated to have variable effects on the activity of a number of enzymes(12,13).

Summary. An overnight fast resulted in decrease in weight of liver of rats and mice

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[†] The spectrophotometer was purchased under grant from Am. Cancer Soc.

TABLE I. Effect of Homogenizing Medium and Nutritive State on Aspartic-Glutamic Transaminase Activities of Tissues.*

Homogenizing medium	Rat			Mouse		
	Liver	Kidney	Heart	Liver	Kidney	Heart
	u/g					
			Fed			
Water	143	97	200	236	118	298
Buffer†	128	73	263	152	88	350
Diff., %‡	–11	–25	+32	–35	–25	+17
			Fasted			
Water	156	92	196	316	119	296
Buffer†	128	83	263	205	92	360
Diff., %	–18	–10	+33	–35	–24	+21

* Male rats, Sprague-Dawley, 80 days old, 310 g body wt. Male mice, Holtzman, 220 days old, 44.6 g body wt. 5 animals/group. Significant changes are underlined. The same homogenates were used for both enzymes (Table II).

† .05 M potassium phosphate, pH 7.4.

‡ Mice (National Lab., 2 mo old) gave differences of –31, –29 and +31 for the respective tissues.

TABLE II. Effect of Homogenizing Medium and Nutritive State on Alanine-Glutamic Transaminase Activity of Tissues.*

Homogenizing medium	Rat			Mouse		
	Liver	Kidney	Heart	Liver	Kidney	Heart
	u/g					
			Fed			
Water	68	5.0	6.4	119	14.2	9.4
Buffer	68	4.4	6.3	121	9.9	8.8
Diff., %†	0	–11	–2	0	–30	–6
			Fasted			
Water	49	4.7	6.3	135	14.3	10.1
Buffer	59	3.5	6.2	122	11.3	8.8
Diff., %	(+20)	–25	–2	–9	–21	–13

* See Table I. Significant changes are underlined.

† Mice (Holtzman, 2 mo old) gave differences of: –6, –16, and 0% for the respective tissues.

with a proportionate decrease in their aspartic- and alanine-glutamic transaminase activities except for the aspartic-glutamic transaminase of mouse liver which maintained its total activity. Homogenates of liver and heart prepared in water had the same alanine-glutamic transaminase activities as those prepared in 0.05 M potassium phosphate (pH 7.4) but the kidney enzyme was lower in buffer. The aspartic-glutamic transaminase activities of liver and kidney were higher in water homogenates while that of the heart was higher in buffer.

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Cyst Formation *in vitro* by *Entamoeba histolytica.* (24019)

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In some laboratories, encystment *in vitro* of *Entamoeba histolytica* seems to occur spontaneously; our own experience has been that this phenomenon occurs but rarely. This difference may be due to the fact that our cultures have been mainly derived from cases of acute, ulcerative, amoebic colitis where the amoebae are, as a rule, large and active and in which cysts are very rarely found. Further, the numerous methods which have been described for inducing cyst formation in cultures by this amoeba have not proved successful by all observers and it is apparent that little is known of the factors underlying the process.

Materials and methods. Recently, by provision of suitable associates, we have been able to culture *E. histolytica* from cases of liver abscess (Freedman, Maddison and Elsdon-Dew(1)). The associates used include *Clostridium welchii, Escherichia coli* and a bacterial complex "M", derived from a previously successful stool culture of *E. histolytica.* Cysts were never seen in the lines cultured with either *Cl. welchii* or *Esch. coli* as associate, but with the "M" complex cysts have been found in small numbers quite regularly. This suggested that there was in the "M" complex some factor or factors which induced amoebae derived from liver to encyst. The "M" complex was therefore separated bacteriologically and the various species found, used singly and in combination, to discover which, if any, were of importance. The species found in the "M" complex are listed in Table I.

Two lines of *E. histolytica* called respectively "A" and "L" and both originally derived from human liver abscesses by provision of *Cl. welchii* as associate were used in the experiments. In some tests the various organisms of the "M" complex were added to an *E. histolytica-Cl. welchii* culture; in others an *E. histolytica-Esch. coli* culture was used. In a few tests, *E. histolytica* freed of its primary associate was used. All tests were made in Locke-Egg tubes.

Results. Table II gives the results.

(i) *E. histolytica* "A" with *Cl welchii* plus test organisms (Table II, column 1).

1. and 2. Confirm that cysts are found with the "M" complex and that by separation of constituent flora the encystment factor has not been lost.

3. Gives results for combinations of 5 of the original 6 organisms. Apparently omission of e or f does not affect cyst formation.

4. Shows that omission of both e and f does not affect encystment.

5. Indicates that in combinations of 3 of the 4 remaining organisms, c is apparently necessary.

6. Shows that in combinations of 2 organisms c is essential for encystment.

7. Gives results for the organisms one at a time. Encystment does not occur, even with c.

8. Shows that c in combination with e gives cysts but not in combination with f.

(ii) *E. histolytica* "A" with *Esch. coli* plus test organisms (Table II, column ii). This column indicates that for all combinations tested the presence of *Esch. coli* as the primary concomitant makes no difference to the results.

(iii) *E. histolytica* "A" plus test organisms (Table II, column iii). This column shows

TABLE I. Bacteria Comprising the "M" Complex.

M.	a.	coliform group
M.	b.	*Idem*
M.	c.	gram negative fusiform bacillus. Anaerobe.
M.	d.	gram positive bacillus. Aerobe.
M.	e.	weakly gram positive bacillus. Aerobe.
M.	f.	streptococcus

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* The Amoebiasis Research Unit is sponsored by: South African Council for Scientific and Industrial Research, Natal Provincial Admin., University of Natal, and U.S.P.H.S.

TABLE II. Cyst Formation with Bacteria of the "M" Complex.

	Bacterial combination with *E. histolytica*	"A" line			"L" line	
		With *Cl. welchii* (i)	With *E. coli* (ii)	No primary associate (iii)	With *Cl. welchii* (iv)	No primary associate (v)
1.	M. complex	+				
2.	M. a,b,c,d,e,f	+			+	
3.	M. a,b,c,d,e	+		—	+	+
	M. a,b,c,d, f	+		+	+	+
	M. a,b,c, e,f	—			+	+
	M. a,b, d,e,f	—			+	—
	M. a, c,d,e,f	—		—	+	—
	M. b,c,d,e,f	—(2)*		—	+	
4.	M. a,b,c,d	+(2)	+	—	+	+
5.	M. a,b, d	—				
	M. a,b,c	+	+	—(2)	+	—
	M. a, c,d	+	+	—	+(2)	—
	M. b,c,d	+	+	—(2)	+	—
6.	M. a,b	—				—
	M. a, c	+(2)	+		+	+
	M. b,c	+	+	—	+	+
	M. c,d	+	+	—	+	
	M. b, d	—				—
	M. a, d	—				
7.	M. a	—(2)		—		
	M. b	—(2)				
	M. c	—(2)		—		
	M. d	—(2)				
	M. e	—				
	M. f	—				
8.	M. c, e	+	+		+	
	M. c, f	—		—		—
	M. d,e				—	

+, cysts present; —, cysts absent. * Except where stated one test only was done.

effect of removal of the primary concomitant by passage through the tissue-bearing medium of Shaffer, Sienkiewicz and Washington(2). Here the results were disappointing in that the only success was attained with the combination a,b,c,d,f.

(iv) *E. histolytica* "L" with *Cl. welchii* plus the test organisms (Table II, column iv). With "L" amoebae and *Cl. welchii* as primary associate the results were similar to those with "A" but encystment occurred here with *all* combinations of 5 organisms.

(v) *E. histolytica* "L" plus the test organisms (Table II, column v). With "L" amoebae and no primary associate, the results differed from those with *Cl. welchii* in that no cysts were found in several instances (3 and 5).

Conclusions. 1) A search for the encystment promoter amongst the organisms comprising a bacterial complex "M" which fosters cyst formation *in vitro* of *E. histolytica* has been made. 2) From study of one line of amoebae "A" it appeared that organism c was essential, but that no one organism (including c) could by itself promote cyst production. Some balance or combination of products or conditions appears to be the requirement. There is a suggestion that one organism (f) may in some way be antagonistic to cyst formation. 3) Another line of amoebae "L" gave similar results to "A" but certain additional combinations of 5 organisms gave cysts. This may indicate a strain difference but, because of the small numbers of cysts found in all tests, we feel that the observation requires verification.

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Selectivity of Heparin-Latex Method and Sensitized Sheep Cell Test for Rheumatoid Serums.* ((24020)

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Since the agglutinating property of serums from patients with rheumatoid arthritis was put to use for diagnostic purposes, this procedure has undergone many changes. The hemolytic streptococci used in the first method(1) were replaced by sensitized sheep erythrocytes(2-5), collodion particles(6), polystyrene globules(7), or bentonite(8). In the original method using polystyrene latex(7), particles of uniform diameter were suspended in Fraction II of human serum(9), then incubated and centrifuged. Agglutination of latex by rheumatoid serum was considered a positive reaction. Gofton *et al.*(10) reported recently that latex particles coated with 1% solution of certain polysaccharides, instead of Fraction II, are also agglutinated by serums from individuals with rheumatoid arthritis. In the present investigation, a modified method using 0.5% heparin as sensitizing agent for latex particles is compared with the standard sensitized sheep cell test(5).

Materials and methods. A polystyrene latex suspension, with globules measuring 0.81 μ diameter and containing 11.2% solids,† was diluted in ratio of 1:10 with distilled water and filtered through Whatman No. 40 filter paper. The borate buffer was prepared by dissolving 6.184 g of boric acid in distilled water, adding 8 ml of 1 N sodium hydroxide and making final volume 2000 ml. The pH was adjusted to 7.9 and 17 g sodium chloride was added. Heparin was purchased from commercial source.‡ Serums from patients with rheumatoid arthritis were obtained at Arthritis Clinic of Grace-New Haven Community Hospital and from private patients of one of the authors (G. de F.). Some samples were stored to 12 months prior to testing. Control serums, furnished by Grace-New Haven Community Hospital, came mostly from different departments. Twelve control samples were obtained from healthy personnel from Yale University School of Medicine. The agglutinating procedure was carried out in 9 progressive dilutions. The first tube contained a serum dilution of 1:20 (1 ml) and progressive 2-fold dilutions were prepared to the ratio of 1:5120. A control tube containing buffer only was included for each serum. To all tubes was then added 1 ml of heparin-latex suspension, prepared by mixing 9.8 ml of buffer, 0.1 ml of 1:10 latex suspension and 0.1 ml 0.5% heparin in buffer. The tubes were then shaken 5 minutes on mechanical shaker, incubated in water bath at 56°C 90 minutes, and, after cooling, centrifuged at 2500 rpm 5 minutes. In positive tests, the latex particles coagulated to fine, sharply defined granules with a slightly turbid or clear supernatant. In a few instances, a fern-like pattern of precipitate was seen on wall of tube. Amorphous, gelatinous masses were sometimes observed in tubes with control sera. They differed, however, distinctly from the sharp granulation in the positive tests. Agglutination appeared in great majority of tests between titers 1:20 and 1:320 and, with few exceptions, was present to highest dilution used, *e.g.* 1:5120. A few serums tested with higher dilutions showed endpoint to be in the 1:10000 to 1:20000 range of the titer. In triplicate determinations on the same serums, the initial agglutination titer differed one dilution only and the coagulum was present to highest titer used. Several tests were made using 1% heparin-latex and uncoated

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† Kindly supplied by Dr. V. K. Rowe, Dow Chemical Co., Midland, Mich.

‡ Organon, Orange, N.J.

TABLE I. Results of 3 Agglutination Tests with Rheumatoid and Control Serums.

Diagnosis	No. of serums	0.5% heparin & latex Pos	0.5% heparin & latex Neg	Sensitized sheep cells Pos	Sensitized sheep cells Neg	No. of serums	Latex only Pos	Latex only Neg
Definite rheumatoid arthritis								
Stage 1	4	2	2	1	3			
2	8	6	2	3	5			
3	47	42	5	30	17			
Total	59	50	9	34	25	42	28	14
Possible rheumatoid arthritis	9	4	5		9			
Juvenile " "	2		2		2			
Psoriasis	3	2	1	2	1			
Spondylitis	5	1	4		5			
Lupus erythematosus	2	2		2		2	2	
Non-rheumatoid patients	78	2	76		78	11		11
Healthy subjects	12		12		12	12		12

latex particles (without heparin). The latex suspension for the latter test was prepared by mixing 9.9 ml of buffer and 0.1 ml of 1:10 latex suspension.

Results. On preliminary testing, the latex fixation test, using 1% heparin as coating agent(10) gave a high proportion of positive reactions with control sera. By decreasing the heparin concentration to 0.5%, these positive reactions could be eliminated and the test still retained a high sensitivity. The results obtained with this modified procedure are given below.

Serums from 59 patients with definite peripheral rheumatoid arthritis were tested with both sensitized sheep cell method and 0.5% heparin-latex procedure. These individuals satisfied the Amer. Rheumatism Asso. criteria for "definite rheumatoid arthritis" and were further subdivided into stages of the disease. Also included were 9 patients with "possible rheumatoid arthritis," 5 spondylitics, 2 with juvenile arthritis, and 3 with rheumatoid arthritis and psoriasis. Seventy-eight serums from non-rheumatoid patients and 12 samples from healthy subjects served as controls. A number of both rheumatoid and control serums was also tested with uncoated latex suspension (without heparin). The overall results are summarized in Table I.

Results with sensitized sheep cells are given in units corresponding to *last* dilution still showing positive reaction. In the heparin-latex test, the *first* dilution showing agglutination was selected, as the reaction was present in most cases up to the highest dilution used, *e.g.* 1:5120.

With one exception, the heparin latex test was positive in all samples which were also positive with sensitized sheep cells. In 8 cases, both heparin-latex and sensitized sheep cells were negative, and in 17 samples the latex-heparin test was positive, sensitized sheep cells negative. The high proportion of negative tests with sensitized sheep cells can be explained partly by the fact that many of these individuals were in clinical remission. Of the 2 control groups, the 12 sera of healthy subjects were negative with all tests. The 78 samples from the Serological Laboratory showed 76 negative and 2 positive tests with 0.5% heparin-latex and they were all negative with sensitized sheep cell test. Of 42 rheumatoid serums tested by latex alone, two-thirds were positive, a higher proportion of positives than reported by Singer and Plotz (7) or Gofton *et al.*(10). Table II gives the results of 22 rheumatoid arthritis serums treated with all 3 tests. With latex only, the agglutination started mostly in tubes with *higher dilutions* and was usually present to 1:5120 titer.

Discussion. The exact mechanism of the agglutination reaction is not known at present. There is an increasing body of evidence, however, suggesting that the polysaccharides either participate directly in the reaction or at least affect it. Treatment of synovial fluids from rheumatoid patients with hyaluronidase was reported to convert atypical hemagglutination patterns to typically positive ones(11). The results of Gofton *et al.*

TABLE II. Comparison of Titers Shown by 22 Individual Serums when Tested by the 3 Methods.

Sensitized sheep cells	0.5% heparin & latex*	Latex only*
256	20	640
128	20	640
256	20	640
Neg	160	160
512	20	320
Neg	80	160
512	20	20
512	160	320
128	20	320
128	320	640
128	20	20
256	160	160
256	80	640
256	40	80
128	20	1280
256	20	20
128	80	640
128	320	640
256	20	640
256	20	320
128	80	160
256	40	160

* First dilution showing agglutination.

(10) and the present findings indicate that certain polysaccharides in system with polystyrene particles of definite size can bring about, or at least enhance, agglutination of latex particles by rheumatoid serums. A high proportion of positive reactions observed with non-sensitized particles suggests that the effect of some of the "coating" agents may be catalyst-like in nature and that possibly systems can be developed which would agglutinate without external sensitizers.

Summary. Results of an agglutination test using polystyrene particles sensitized with 0.5% heparin are described. Sensitivity and selectivity of this system for rheumatoid serums compares favorably with those of the standard sensitized sheep cell test method. A high proportion of rheumatoid serums was found to give positive reactions with non-sensitized latex particles.

The authors are indebted to Miss Letitia Feliciano for technical assistance and to Miss Juliet Drabkin for serums from Serological Laboratory.

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Effect of Adrenalectomy on Albumin-Palmitate-1-C^{14} Oxidation.* (24021)

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Studies have suggested that the adrenal glands play an important role in fat metabolism. The effect of adrenalectomy on ketosis (1-6) has been used as an indicator of the role of adrenals in fat catabolism but the results of such studies have been contradictory. Recent work(7,8) with isotopic tracers indi-

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cated that fat oxidation is enhanced by adrenalectomy.

In this study a new approach has been applied to the question of oxidation of fat by the adrenalectomized rat based on findings of Gordon and Cherkes(9) and Havel and Fredrickson(10). Their results indicated that the small quantity of non-esterified fatty acid complexed to serum albumin (NEFA) plays a major role in fatty acid metabolism. Indeed, it was suggested that NEFA is the transport form of fatty acids that accounts for movement of fatty acid from fat depots to other metabolically active tissues during fasting. Recently Felts(11) and McCalla *et al.*(12) have prepared a palmitic acid-1-C^{14}-albumin complex. It was found that surviving rat liver slices oxidized this compound to $C^{14}O_2$ at high rates(11) and that, following intravenous administration to rats, the intact animal rapidly converted it to $C^{14}O_2$(12). Since NEFA probably is the metabolically important form of blood fatty acids, a study of oxidation of this lipid complex by adrenalectomized animals should provide information regarding the role of adrenal hormones on fat catabolism.

Methods. Mature male rats of Wistar strain were kept at 25°C for 10 days before surgery and ate the following diet: 25% casein, 10% lard, 51% glucose, 3% salt mix (13), 5% cellulose, 6% brewers yeast, 0.04% cod liver oil concentrate, 0.01% α-tocopherol. Then the rats were either bilaterally adrenalectomized or sham-operated. Metabolic studies were carried out 11-20 days after surgery. The adrenalectomized rats drank 0.9% NaCl solution during the post-operative period. The albumin-palmitate-1-C^{14} was prepared by the method of Felts(11).§ The albumin-palmitate-1-C^{14} solution (½ ml containing 1.3 micromoles of palmitic acid) was injected intravenously into rats that were lightly anesthetized with ether and that had been fasted 12-18 hours. The rats were then immediately placed into a specially designed metabolism cage, ventilated continuously with CO_2-free air. The $C^{14}O_2$ expired by the rat during the experiment was collected over half-hour intervals for 2 hours by the method of Masoro *et al.*(14) and the $BaC^{14}O_3$ thus formed was analyzed for C^{14} content and specific activity by the method of Entenman *et al.* (15). The total amount of $BaCO_3$ in the sample was determined gravimetrically. At the end of the experiment, the sham-operated rats were discarded but adrenalectomized rats were placed on Purina chow and tap water. Only those rats that died in a few days after being placed on tap water were considered to have been completely adrenalectomized. Data from adrenalectomized rats that did not die were discarded.

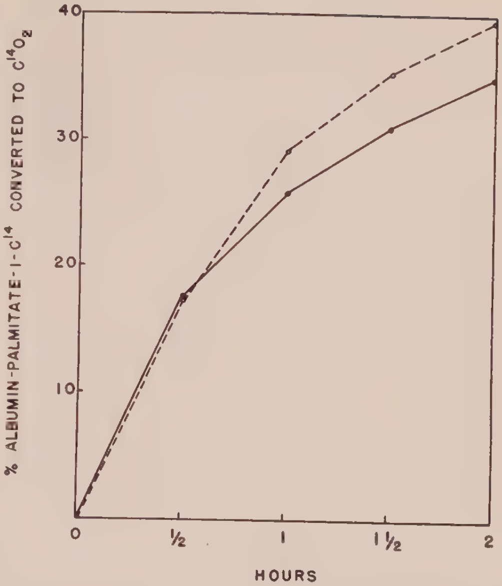

FIG. 1. Effect of adrenalectomy on oxidation of albumin-palmitate-1-C^{14}. Each point represents avg value obtained from 5 rats. Solid line refers to sham-operated rats (avg body wt, 195 g) while hatched line refers to adrenalectomized rats (avg body wt, 200 g). Data are recorded in terms of cumulative % of palmitate-1-C^{14} converted to $C^{14}O_2$.

Results. The per cent of injected albumin-palmitate-1-C^{14} converted to $C^{14}O_2$ in ½, 1, 1½ and 2 hours by sham-operated and adrenalectomized rats is recorded in Fig. 1. Since the work of Havel and Fredrickson(10) indicates that the half-life of NEFA is far less than 5 minutes, it is probable that the

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§ This method consists of adding sodium salt of labeled fatty acid to neutral solution of albumin (Crystalline Bovine serum albumin, Armour). By this method 6 moles of fatty acid/mole of albumin may be complexed at physiological pH.

TABLE I. Albumin-Palmitate-1-C^{14} Oxidation in Normal and Adrenalectomized Rats.

	Rat No.	Body wt, g	% inj. C^{14} converted to $C^{14}O_2$ in 1st half hr	mMoles of CO_2 expired in 1st half hr
Sham-op.	5	260	16.3	7.75
	6	240	18.4	
	19	140	20.0	5.22
	32	160	11.8	4.36
	40	190	20.8	6.18
Adrenalectomized	3	220	15.7	5.98
	12	170	19.6	4.37
	28	210	17.9	6.86
	38	190	18.7	6.90
	37	210	15.2	7.32

$C^{14}O_2$ evolution in the first half-hour would give the best index of rate of NEFA oxidation. The sham-operated rats converted 17.5% of the injected albumin-palmitate-1-C^{14} to $C^{14}O_2$ in the first half-hour, while the adrenalectomized rats converted 17.4% to $C^{14}O_2$ during the same time. The data suggest that the fasting rat's ability to oxidize NEFA is not influenced by adrenalectomy. At later time intervals the adrenalectomized rats formed somewhat more $C^{14}O_2$ than sham-operated rats. However, the differences are not statistically significant. Moreover, the $C^{14}O_2$ yields at later intervals are influenced by a variety of metabolic processes such as ability to store fatty acids in ester form, and thus these time intervals are less reliable indices of the rate of NEFA oxidation than the earliest time interval studied.

Since the first half hour of metabolism is probably the best index of rate of NEFA oxidation, the data for each rat during this interval of time are presented in Table I. It is apparent that no relationship exists between body weight and per cent of albumin-palmitate-1-C^{14} converted to $C^{14}O_2$, in either the adrenalectomized or sham-operated group of rats. Furthermore no relationship exists between total metabolic rate (as measured by millimoles of CO_2 expired) and per cent of albumin-palmitate-1-C^{14} converted to $C^{14}O_2$. This is particularly well demonstrated in adrenalectomized rats No. 3 and 37. Both animals have about the same body weight; rat No. 37 produced about 25% more CO_2 than rat No. 3, while rat No. 3 formed more $C^{14}O_2$ than rat No. 37 (Table I).

Discussion. The role of the adrenal gland in fat catabolism has been the subject of much study, and work on ketosis of adrenalectomized rats(1-6) has led to conflicting conclusions. Much of this confusion has probably resulted from the fact that ketosis is a state of balance between ketogenesis and ketolysis and therefore is not a good measure of rate of fatty acid oxidation. Recent studies(7,8) with isotopes have somewhat clarified the situation. In our study the rapidly turning over NEFA fraction(10) of plasma lipids was labeled by intravenously administering artificially prepared albumin-palmitate-1-C^{14}. It is apparent from the results that adrenalectomy neither increases nor decreases ability of tissues of the fasting animal to oxidize the NEFA that is so rapidly picked up from the blood. Although Welt and Wilhelmi(7) presented evidence that the turnover of biosynthesized body fat is greater in the fed adrenalectomized animal than in the fed normal, an explanation of their results may lie in an altered transport or storage, or both, of the biosynthesized fat of the fed animal.

Perry and Bowen(8) presented evidence suggesting that liver slices from adrenalectomized rats oxidize octanoate-1-C^{14} more rapidly than slices from control rats. However, it is known that enzymes(16) involved in activating fatty acids are not the same for short-chain and long-chain fatty acids and therefore differences in findings between studies using short-chain acids and those using long-chain acids might be expected.

The $C^{14}O_2$ yield from albumin-palmitate-1-C^{14} in the fasting rat is not related to either body weight or total CO_2 production of the rat. This finding is clearly of importance, but at present the interpretation is obscure.

Summary. Oxidation of albumin-palmitate-1-C^{14} (NEFA) to $C^{14}O_2$ was studied in sham-operated and adrenalectomized rats fasted for 12-18 hours. The $C^{14}O_2$ yield was approximately the same in both animal groups. The conclusion is drawn that ability of rat tissues to oxidize long-chain fatty acids is not affected by adrenalectomy. The $C^{14}O_2$ production from albumin-palmitate complex is not related either to size of animal or to endo-

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Sex Difference in Induction of Periportal Fatty Liver by Methionine Deficiency in the Rat.*† (24022)

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Ethionine, the ethyl analogue of methionine, was previously reported to induce periportal fatty liver within 10 to 12 hours after intraperitoneal administration to female but not male rats(1,2,3). Administration of testosterone propionate or other androgens to susceptible animals protected against this effect of ethionine(2,4). Simultaneous methionine administration, but not other amino acids, also protected the animals(1,5,6). In contrast, choline showed no protective effect (1,6). These results suggested that ethionine may be producing a fatty liver by interfering with some metabolic role of methionine. This would imply that methionine plays another role in prevention of some fatty livers, in addition to its ability to contribute methyl groups for synthesis of choline. The protective role of methionine would appear to be linked in some as yet unexplained manner with the action of androgens. However, before this suggestion could be seriously considered, it was important to observe whether animals given methionine devoid diets would develop fatty liver with lobular pattern and sex difference similar to that found with ethionine.

Induction of fatty liver by feeding rats diets devoid in methionine and adequate in choline has been observed(7) but lobular distribution of excess lipid and presence or absence of sex difference have not been reported heretofore. In the present study, liver lipid content and histologic distribution of excess lipid was determined in male and female rats, force-fed methionine devoid and complete diets. To determine specificity of changes observed with methionine deficiency, similar animals were force-fed a diet devoid in another essential amino acid, threonine. This amino acid was selected because diets devoid in threonine induced periportal fatty liver in immature rats (8).

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‡ Life Insurance Medical Research Fellow, 1956.

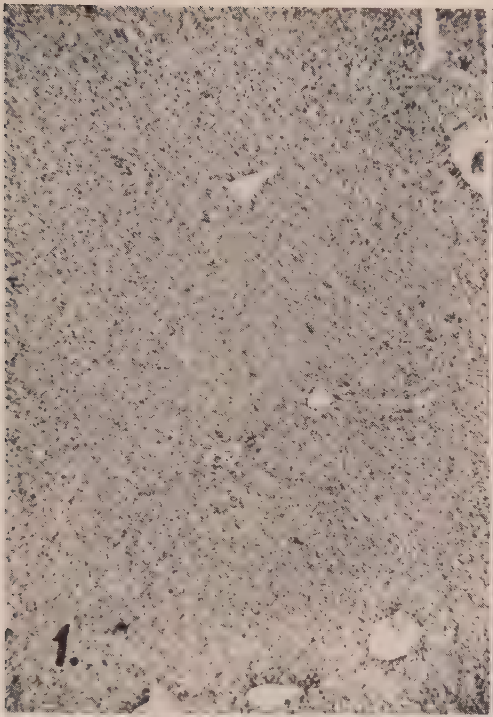
TABLE I. Liver Weight and Lipid in Male and Female Rats Force-fed Methionine Devoid or Complete Diets.

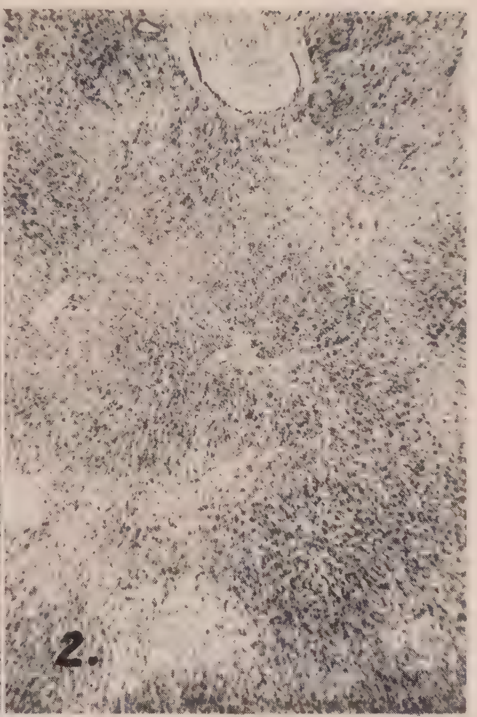
					Liver lipid			
Diet	Sex	No. of rats	Liver wet wt, g/100 g body wt		g/100 g liver		mg/liver/100 g body wt	
Complete	♂	10	4.28	.17*	6.6	.6*	275	28*
Methionine devoid	♂	9	4.23	.15†	7.1	.5†	292	14†
Complete	♀	13	4.21	.14	6.9	.7	289	26
Methionine devoid	♀	12	4.76	.20§	11.3	.9‡	587	98‡

* Mean ± stand. error of mean. † $P > .05$ (not significant). ‡ $P < .01$ (highly significant). § P between .05 and .01 (probably significant).

Materials and methods. White rats of both sexes of Wistar (Carworth Farms) and Sprague-Dawley strains, weighing 130 to 240 g, were maintained on Purina Laboratory Chow until beginning of experiments. The animals were placed in individual cages in air conditioned room and after overnight fast, were force-fed twice daily at 8:30 a.m. and 4:30 p.m. The method of Shay and Gruenstein(9) for tube feeding was used. The basal diet was similar to that of Forbes and Vaughn(7). It was composed of: essential amino acids 9.23%, non-essential amino acids 9.69%, salt mixture 4%, vitamin sucrose mixture 5%, corn oil 5%, cod liver oil 1.5%, and sucrose 65.58%. The essential amino acids consisted of: L-lysine HCl 1.24%, L-arginine HCl 0.75%, DL-tryptophan 0.20%, DL-phenylalanine 0.90%, DL-leucine 1.60%, DL-isoleucine 1.0%, DL-valine 1.4%, L-histidine HCl 0.54%, DL-methionine 0.67% and DL-threonine 1%. The non-essential amino acids consisted of: L-glutamic acid 2.0%, DL-serine 0.50%, glycine 0.7%, DL-tyrosine 2.8%, L-cystine 0.2%, L-proline 0.9%, L-asparagine monohydrate 1.39%, and DL-alanine 1.2%. The vitamin mixture contributed the following amounts in mg/100 g of diet: Thiamine HCl 0.25, riboflavin 0.5, pyridoxine HCl 0.25, calcium pantothenate 2, nicotinamide 1, choline chloride 100, biotin 0.01, folic acid 0.1, inositol 10, 2-methyl, 1, 4-naphthoquinone 0.1 and Vit. B_{12} 0.01. The mineral mixture was that of Hegsted *et al.*(10). The devoid diets contained added sucrose in place of the missing amino acid, methionine or threonine. The diet mix was blended with water so that 1 ml of diet mixture contained 1 g of diet. One group of animals was tube fed the complete ration while other groups were given the devoid diets. In some experiments, rats received from ½ to 2 days of force-feeding of complete diet before beginning experimental diet while in others, the experimental diet was fed after initial overnight fast. No differences were observed between the 2 groups. Each rat received a total daily intake of from 0.7 to 0.9 g of diet/10 g of initial body weight. Animals were allowed free access to water. In the methionine devoid experiments, rats were sacrificed at 3, 4, or 6 days after beginning the experiment. Since the results in each group were the same at different time intervals, the data were combined. In experiments with threonine devoid diet, the rats were killed after being on diets for 3 days. Animals were anesthetized with ether and sacrificed by exsanguination 18 hours after last feeding. For total liver lipid analysis, aliquots of liver were dried by grinding with anhydrous sodium sulfate and extracted with chloroform for 24 hours. The residue was extracted with petroleum ether and lipid was determined gravimetrically after evaporation of solvent. Pieces of liver were fixed in Bouin's solution and in 10% formalin. Paraffin sections were stained with hematoxylin-eosin and frozen sections with Sudan IV.

Results. Force-feeding of methionine devoid diet to adult rats rapidly induced fatty liver in females but not in males (Table I). In females on methionine devoid diet, livers showed average of 13% increase in total liver weight, a 64% increase in % lipid and 103% increase in total lipid/100 g body weight. These differences are statistically significant at high level of probability. In contrast, in the male, livers showed 1% decrease in liver weight, only 8% increase in % lipid and 6% increase in total lipid. These changes are not





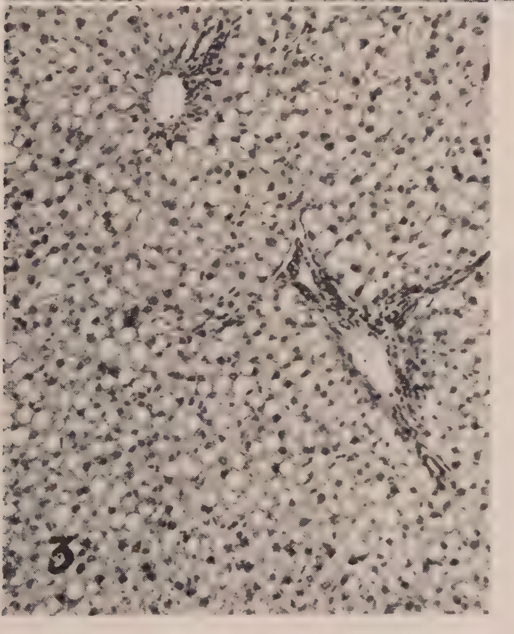


FIG. 1. Liver of female rat on complete diet, showing virtual absence of stainable lipid. Livers from male rats on complete diet and on methionine devoid diet showed the same appearance. Sudan IV; × 30.

FIG. 2. Liver of female rat on methionine devoid diet. Note periportal distribution of lipid. Compare with Fig. 1. Sudan IV stain; × 30.

FIG. 3. Liver of female rat on methionine devoid diet. Lipid vacuoles predominantly in periportal area. Hematoxylin and eosin; × 100.

significant. The average daily changes in body weight during experiments were as follows: Male controls, +3.1 g : male methionine devoid, –0.7 g : female controls, +1.7 g : and female methionine devoid, +0.7 g. These changes in body weight were small in comparison to total weights of animals and therefore do not influence to any significant degree the liver lipid values when expressed on the basis of liver weight/100 g body weight.

The sex difference found by chemical determination of liver lipid was confirmed by study of histologic sections (Fig. 1 to 3). When

TABLE II. Liver Weight and Lipid in Adult Rats Force-fed Threonine Devoid or Complete Diets for 3 Days.

Diet	Sex	Liver wet wt, g/100 g body wt			Liver lipid, mg/100 g body wt		
Control	♀	(4)*	4.03	.28†	(3)*	248	39†
Threonine devoid	♀	(3)	4.69	.18‡	(2)	440	21§
Control	♂	(6)	3.77	.13	(6)	256	13
Threonine devoid	♂	(6)	5.44	.38‖	(6)	413	25‖

* No. of animals in parentheses.
† Mean ± stand. error of mean.
‡ P >.05 (not significant).
§ P between .05 and .01 (probably significant).
‖ P <.01 (highly significant).

liver lipid was increased, its distribution was consistently periportal.

In contrast to results with methionine devoid diet, the livers of both males and females fed threonine devoid diet showed increase in liver lipid (Table II). Although the rise in liver lipid was statistically significant in both sexes, the increase in the male was significant at a higher level of probability.

Discussion. Our results clearly show that female rats, but not males, develop fatty liver when force-fed choline containing diets devoid of methionine. Excess lipid is periportal in distribution, in contrast to centro-lobular localization of fat in animals fed choline deficient low-methionine diets(11). Animals force-fed threonine devoid diets also develop a periportal fatty liver but without a sex difference.

Using a few adult rats, Spector and Adamstone(12) reported no sex difference in periportal fatty liver induced by force-feeding tryptophan deficient diets. Using force-fed tryptophan, isoleucine or phenylalanine devoid diets, Samuels and coworkers(13,14) also reported induction of periportal fatty liver in young adult male rats. Thus, although periportal fat deposits have been produced by a variety of single amino acid deficiencies, a sex difference has not been reported to occur with any amino acid except methionine.

That both methionine devoid diets and ethionine administration produce fatty livers in female but not in male rats lends further support to the thesis that ethionine acts by producing a functional methionine deficiency. Since fatty livers induced by ethionine or by methionine devoid diets are periportal rather than centro-lobular and are not prevented by choline, methionine must have some additional role in prevention of some forms of fatty liver, aside from its ability to contribute methyl groups for synthesis of choline. Since it has been fairly well established that the sex difference in production of fatty liver by ethionine is due, at least in part, to the presence of androgens in the male(2,4), additional metabolic property of methionine in fatty liver appears to be related in some as yet unknown manner with the action of androgens. Further support for this relationship is found in the common "protein sparing" action of both methionine(15) and androgens(16) and the sex difference, probably also androgen mediated, in the inhibition by ethionine of protein synthesis in the liver *in vivo* and *in vitro* (17, and unpublished). The observed correlation between inhibition of hepatic protein synthesis and subsequent appearance of fatty liver in rats given ethionine(17) suggests that the effects of methionine and androgens in preventing ethionine-induced fatty liver may be concerned with some common or interrelated effect of each on protein metabolism.

Summary. 1. Force-feeding of purified choline-containing diets devoid of methionine for 3 to 6 days induces fatty liver in adult female rats, but not in adult male rats. Excess lipid accumulates in the periportal region of liver lobule. 2. Force-feeding of threonine-devoid diet for 3 days to adult rats also induces periportal fatty liver but without a sex difference. 3. Occurrence of the same sex difference and the same lobular distribution of fat in the liver by force-feeding methionine devoid diets and by injection of ethionine lends further support to the thesis that ethionine exerts some of its effects through interfering with metabolic role of methionine. 4. Since choline does not protect liver against the effects of methionine deficiency, it appears that methionine must play some role in regulation of lipid content of liver aside from its contribution of methyl groups for synthesis of choline. It is suggested that this role of methionine is related in some as yet unknown manner with the action of androgens.

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Cholinesterase Activity and Potassium Permeability in Human Erythrocytes. (24023)

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Since Dale's(1) suggestion that an enzyme was present in blood which brought about destruction of acetyl choline, and subsequent work which led to clarification of the nature of this enzyme as a specific acetyl cholinesterase(2-5), its exact role in metabolism of the erythrocyte has remained obscure. Lindvig, Greig, and Peterson(6) found that acetyl choline decreased the net exchange of sodium and potassium of human erythrocytes suspended in bicarbonate buffer, and also delayed hemolysis which was apparently due to increased intracellular cation and water. These processes were reversed by physostigmine. Production by normal erythrocytes of a substrate for cholinesterase, demonstrated by Holland and Greig(7), could necessitate a means for its removal upon completion of its function. Thiele and Pennell(8) confirmed the production of cholinesterase substrate in erythrocytes, which, at –1.5 - 0°C, greatly exceeded its hydrolysis. They postulated a cholinesterase-substrate-dextrose relationship important in synthetic and hydrolytic functions, and suggested also a contractile function of acetyl choline, alternately contracting and relaxing the fibrous structural proteins of the cell as a means of regulating membrane permeability. Taylor, Weller and Hastings(9), using radioactive potassium and calculating its concentration as the resultant of 2 exchange rates, showed a loss of the ion from human red cells when exposed to cholinesterase inhibitors in concentrations approximately 100 times that necessary to inactivate completely the enzyme. The relationship between enzyme inhibition and potassium loss was not readily apparent. Goodman, Marrone and Squire(10) found that the *in vivo* potassium gradient was unrelated to erythrocyte cholinesterase activity.

The following report describes our attempts to demonstrate altered permeability to potassium in human erythrocytes in the presence of acetyl choline, and cholinesterase inhibitors diisopropylfluorophosphate and physostigmine

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TABLE I. Serum and Cell Potassium Concentrations during Storage with Physostigmine, DFP and DFP + AcCh. Concentration of physostigmine = 5×10^{-6} molar; concentration of DFP = 0.01 mg/ml; concentration of AcCh = 3.6×10^{-3} molar. Values are in milliequivalents/l of serum or packed cells.

		Days								
		0	2	3	4	7	8	10	12	15
Blood + physostigmine	Cells	109.0		101.8		85.0		80.6		83.9
	Serum	4.3		8.5		10.9		13.1		16.0
Control	Cells	105.3		91.8		87.2		77.7		82.6
	Serum	4.2		9.0		11.5		13.8		16.9
Blood + DFP	Cells	91.9			84.3		77.9		70.9	67.8
	Serum	4.5			13.3		20.2		26.8	29.6
Control	Cells	93.4			86.3		81.2		72.4	70.6
	Serum	4.0			12.2		17.9		25.4	27.4
Blood + DFP + AcCh	Cells	111.3	102.8							
	Serum	4.1	7.8							
Control	Cells	106.5	100.3							
	Serum	4.4	8.6							

sulfate at 4° and 37.5°C.

Materials and methods. Normal blood was drawn aseptically from donors into either acid-citrate-dextrose (ACD) solution (20 ml/100 ml blood) or heparin sodium (2000 USP units/100 ml blood). Two separate aliquots of 250 ml each were drawn from each donor for storage experiments in ACD at 4°C. Acetyl choline chloride (Merck) (AcCh) was dissolved in sterile Parpart's buffered saline, pH 7.4(11). Physostigmine sulfate (Merck) solution was prepared in Parpart's saline and filtered through a Seitz filter. Diisopropylfluorophosphate (Technical Operations, Inc.) (DFP) was autoclaved in sealed capillary tubes; solution was effected by crushing capillaries in sterile Parpart's saline. Drugs in 5 ml saline were added to blood with sterile, cotton-plugged pipettes under ultraviolet hood. Sterile saline was added to control bottles. Aliquots for analyses were removed aseptically with an 18 gauge needle and 20 ml syringe. Cultures of stored blood at conclusion of each experiment showed no growth in 48 hours on beef infusion agar and broth with 5% dextrose, thioglycollate broth, or trypticase soy broth. Cholinesterase activities and AcCh concentrations were determined by the method of Hestrin(12). Potassium analyses were done on Beckman model DU spectrophotometer with flame attachment. Experiments at 37.5°C were done in 25 ml Erlenmeyer flasks with 10 ml heparinized blood and the drug(s) added in 0.53 ml 5% dextrose. Flasks were covered with Parafilm (Marathon Corp.) with pinhole to allow gas exchange but retard evaporation, and shaken gently at 90 oscillations/minute on a rack mounted in Warburg apparatus. Hematocrit values were determined by the method of Wintrobe. These values did not change significantly in any sample during the experiment.

Results. Table I shows serum and cell potassium concentrations during storage at 4°C with physostigmine, DFP and DFP + AcCh. Periodic cholinesterase assays showed the enzyme to be completely inhibited by DFP, and about 80% inhibited by physostigmine, at concentrations used. This latter figure is, however, probably lower than degree of inhibition actually existent in stored blood, due to dilution (100 fold) of the enzyme during assay with concomitant partial dissociation of the enzyme-drug complex. The degree of inhibition remained essentially constant during the study. The experiment with DFP + AcCh was terminated at 48 hours due to non-enzymatic hydrolysis of 90% of the added AcCh at that time.

Table II shows the effect of AcCh, DFP, and DFP + AcCh on re-entry of potassium into cells which had been stored for 24 hours in heparin, prior to incubation at 37.5°C, to permit leakage of potassium into the serum. Table III shows the effects of the same drugs and combinations of drugs on fresh heparin-

TABLE II. Effects of DFP and DFP + AcCh on Re-entry of Potassium into Erythrocytes Stored 24 Hr at 4°C. Temperature of incubation, 37.5°C. Concentration of DFP, 0.01 mg/ml; concentration of AcCh, 3.6 × 10^{-3} molar. Values are in milliequivalents/liter of serum or packed cells.

		0 hr	3 hr	6 hr
Blood only	Cells	112.5	120.6	123.4
	Serum	9.2	6.2	6.6
Blood + DFP	Cells	112.5	124.1	123.0
	Serum	9.2	6.2	6.4
Idem + AcCh	Cells	117.5	123.5	123.2
	Serum	8.8	6.2	6.0
Blood + AcCh	Cells	117.5	125.6	124.1
	Serum	8.4	6.2	6.0

ized cells.

Discussion. The suggestion(6) that erythrocyte cholinesterase may be actively engaged in AcCh hydrolysis for maintenance of normal cell fragility implies a function of the enzyme similar to its role in neural transmission. AcCh produced intracellularly or on the cell surface could be responsible for maintaining a certain status of the membrane with the resultant cation partitioning. In view of the known extracellular site of action of AcCh at the synapse and myoneural junction, if the 2 processes were similar, one would expect a significant decrease in cation concentration gradient upon either inhibition of the enzyme (assuming continued production of cholinesterase substrate) or addition of exogenous substrate, or both. The fact that no changes were apparent over and above control changes in the potassium gradient under these conditions indicates that this cation is not the one primarily involved in the suggested synthetic-hydrolytic system.

TABLE III. Effects of DFP and DFP + AcCh on Escape of Potassium from Fresh Erythrocytes. Temperature of incubation, 37.5°C. Concentration of DFP, 0.01 mg/ml; concentration of AcCh, 3.6 × 10^{-3} molar. Values are milliequivalents/liter of serum or packed cells.

		0 hr	3 hr	6 hr
Blood only	Cells	114.5	115.9	116.0
	Serum	3.0	4.6	5.3
Blood + DFP	Cells	114.8	116.6	115.9
	Serum	2.9	4.6	5.3
Idem + AcCh	Cells	116.0	118.6	117.8
	Serum	2.9	4.5	5.0
Blood + AcCh	Cells	117.5	118.2	119.5
	Serum	2.9	4.7	5.3

Summary. Inhibition of human erythrocyte cholinesterase with or without exogenous AcCh had no effect on loss of potassium from cells during storage at 4°C or during incubation at 37.5°C. The re-entry at 37.5°C of potassium into cells previously stored for 24 hours at 4°C to permit its leakage into the serum was also unaffected.

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Serological Detection of Desoxyribonucleic Acid (DNA) Adsorbed to Formalinized Erythrocytes.* (24024)

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During the past 3 years we have studied the immunology of the Rous sarcoma, a virus-induced tumor of chickens. A virus-containing eluate of tumors prepared by the method of Riley(11), and desoxyribonucleoprotein (DNAP) preparations from tumor and normal chicken muscle prepared by the method described for calf thymus by Mirsky and Pollister(10) have been examined. It has been found that potent precipitin serums prepared against DNAP will not precipitate with corresponding purified protein-free desoxyribonucleic acid (DNA) preparations. This has also been the experience of other workers investigating the serological reactivity of nucleoproteins(8). Cole and Farrell(3) reported the use of formalinized erythrocytes with protein antigens coupled to them, to detect antibodies against the attached proteins. We used formalinized erythrocytes coupled with sulfanilazo groups to measure formation in rabbits of antibody homologous to this hapten. It has been found that DNA will couple to formalin-treated erythrocytes to form an antigen which can be agglutinated by serums prepared against DNAP.

Materials and methods. Phosphate-saline. Sodium chloride, 0.145 M, containing 0.005 M sodium phosphate with final pH of 7.3-7.4 was used. *Formalin-saline.* Formaldehyde, 10 g/100 ml, in phosphate-saline with final pH of about 7.0 was employed. *Coupling solution.* McIlvaine's buffers were mixed with 0.145 M saline to give final pH 4.9. This pH was chosen as optimum after trials at pH 2.9, 3.9, 4.9, 5.9, 6.9, 7.9 and 8.9. *Formalinized erythrocytes.*‡ Human type O blood cells from an outdated blood bank unit were washed 3 times with 4 volumes of phosphate-saline containing 0.5% dextrose, made to a 50% suspension in phosphate-saline and chilled to 0-3°C. This chilled suspension was poured into 4 volumes of formalin-saline solution and stored in refrigerator at 0-3°C. The cells were resuspended once each day for the first week and then remained in refrigerator until used. The cells employed were in formalin-saline solution approximately 6 months prior to use. However, cells have been used successfully after 2-3 weeks of treatment with formalin. *Desoxyribonucleic acid.* DNA from Rous sarcoma cells (tumor DNA) and DNA from normal chicken muscle (normal DNA) were initially prepared as nucleoprotein by molar saline extraction procedure of Mirsky and Pollister(10). The method of Kay, Simmons, and Dounce(6) was then employed to remove the proteins. DNA thus recovered was a white, ropy substance with U.V. adsorption maximum at 260 mμ and an ϵ (P) of approximately 6600 as described by Chargaff(1). The biuret test as reported by Holden and Pirie(4) showed less than 1% protein contamination in the DNA. The N/P ratio was 2.0 and 2.1 for tumor DNA and normal DNA respectively. The antiserum against tumor DNAP and normal DNAP were prepared in rabbits by intravenous injection of antigen in 1 M saline and subcutaneous injection after incorporation into Freund adjuvant (2). *Coupling technic.* Formalinized erythrocytes were washed 5 times in 20-30 volumes of phosphate-saline to remove free formaldehyde. A 10% suspension of erythrocytes was made in the coupling solution and 0.1 mg of DNA (dry wt) was added/ml of cell suspension. This mixture was incubated in a 37°C water bath with mixing every half hour for 4 hours. Erythrocytes were then washed 3 times with phosphate-saline to remove excess DNA and the cells resuspended in phosphate-saline. The supernatant from third washing was negative for DNA as shown by the diphenylamine reaction. The coupled-cells could be stored at 4°C for approximately 4 weeks without loss of reactivity. *Agglutination pattern test.* Selected tubes 14.5 x 75 mm were used in racks having holes of approximately 12 mm diameter in bottom shelf. Each tube contained 0.5 ml of serum dilution in phosphate-saline containing 1:100 normal rabbit serum and 0.1 ml of a 1% cell suspension in phosphate-saline. After 24 hours the test was read by observing the bottoms of tubes in a mirror while the tubes were illuminated from above with a fluorescent lamp. The highest serum dilution which gave a pattern of agglutination clearly distinguishable from normal serum controls was taken as end point. Cole and Farrell(3) and Stavitsky(12) have discussed the problem of interpreting agglutination patterns similar to those observed in this work. The above procedure was repeated 6 times for each antiserum, and the observed end point was the same in each experiment. *Inhibition study.* To obtain further evidence that agglutination depended upon a specific combination of antibody with DNA attached to cells, an inhibition experiment was done with either homologous or heterologous DNA as the inhibiting substance. Before addition of cells, 0.1 ml of 0.5 mg/ml solution of either normal DNA or tumor DNA was added to each tube. After 2 hours incubation at room temperature the coupled-cells were added, and the test completed as described above. Various concentrations of inhibiting DNA were tried, and it was observed that inhibition increased with concentration using 0.01-0.025 mg DNA, but no further increase was observed at concentrations up to 0.1 mg DNA. Therefore, 0.05 mg DNA was employed to insure an excess of inhibiting DNA. *Optical density study of agglutination.* The method of Liener(9) was employed to obtain a semi-quantitative measure of agglutinating capacities of the serums. One ml of a 2% cell suspension was added to 1 ml of a 1:20 dilution of antiserum or a 1:10 dilution of normal rabbit serum. A 1:20 dilution of normal rabbit serum had shown no effect upon coupled or uncoupled cells, and it was felt that employment of a 1:10 dilution would demonstrate any non-specific action, which might be present but not demonstrable at a 1:20 dilution of normal serum. Optical density was read at 620 mμ using a Coleman Jr. spectrophotometer, model 6A. A reading was taken 10 minutes after mixing and every 50 minutes thereafter to 200 minutes. *Enzyme activity.* Once recrystallized desoxyribonuclease (DNA-ase) prepared according to the method of Kunitz(7) was obtained from Worthington Biochemical Corp. (Lot #D564). Trypsin (1:250) was obtained from Difco Laboratories and prepared at concentration of 5 mg/ml in phosphate buffer at pH 8.0. Aliquots of formalinized cells coupled with either normal DNA, tumor DNA, bovine serum globulin (BSG), or sulfanilic acid (SA), as well as uncoupled formalinized cells were exposed to either DNA-ase (0.1 mg/ml) for 24 hours at room temperature or trypsin (0.1 mg/ml) for 1 hour at 37°C. Controls included coupled and uncoupled cells exposed to enzyme diluents under the above conditions. Cells were washed 5 times following treatment and resuspended in phosphate-saline. The resultant cell suspensions were tested as described above for agglutination pattern and optical density change.

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* Some data in this report are included in thesis to be submitted to Graduate School of Indiana University in partial fulfillment of requirements for degree of Doctor of Philosophy.

† Predoctoral fellow of the Nat. Cancer Inst., U.S.P.H.S.

‡ This procedure was designed by Dr. J. S. Ingraham, Dept. of Microbiology, Indiana Univ. Med. Center.

Results. From data in Table I it can be seen that after formalinized erythrocytes are exposed to either tumor or normal DNA they are agglutinated specifically by antiserum prepared against homologous DNAP. Both anti-tumor DNAP and anti-normal DNAP serums cross react in lower titer with heterologous DNA-cells. The settling pattern results in Table I agree with those from rate-of-settling experiments shown in Fig. 1. In this figure antiserum causes cells coupled with the DNA to settle more rapidly than controls. The results, which are free of subjective judgment encountered in interpreting patterns, are in complete agreement with those obtained in the agglutination pattern study.

Specific inhibition obtained in tumor DNA-anti-tumor DNAP, and normal DNA-anti-normal DNAP reactions as shown in Table I, supports the conclusion that there is at least

TABLE I. Titers Observed by the Agglutination Pattern Technic.

Anti-serum	Cells	Inhibiting DNA*	Hemagglutinating titer
Tumor-DNAP	Tumor-DNA		1:16,000
	”	Tumor-DNA	1:500
	”	Normal- ”	1:8000
	Normal-DNA		1:1000
	”	Tumor-DNA	<1:2
	”	Normal- ”	”
	Uncoupled		”
Normal-DNAP	Normal-DNA		1:8000
	”	Normal-DNA	1:200
	”	Tumor- ”	1:4000
	Tumor-DNA		1:2000
	”	Normal-DNA	<1:2
	”	Tumor- ”	”
	Uncoupled		”
NRS†	Tumor-DNA		<1:2
	Normal- ”		”
	Uncoupled		”

* DNA preparation and antiserum were incubated 2 hr prior to addition of cells (.05 mg DNA).

† Normal rabbit serum.

a considerable quantitative difference between tumor and normal DNA preparations. Complete inhibition of cross-reactions by either DNA preparation might be expected to occur even if there were qualitative differences between the 2 DNA preparations. The fact that inhibition was obtained suggests that antibody reacts with the DNA in the absence of a carrier protein. However, it has not been possible to precipitate anti-DNAP serum with purified DNA.

Desoxyribonuclease at concentration employed completely removes serological reactivity of normal DNA-cells (Fig. 2). However, one observes that tumor DNA-cells retain a slight amount of serological reactivity with homologous antiserum. The DNA-ase had no apparent effect upon agglutination pattern and settling rate of BSG-cells or SA-cells in homologous antiserum. Uncoupled cells in normal and the various antiserums were unaffected by this enzyme.

Trypsin at concentration employed did not alter reactivity of DNA-cells; furthermore, it did not remove activity which remained in the homologous tumor DNA-cell system after DNA-ase treatment. However, the amount of trypsin employed was sufficient to remove completely the reactivity of BSG-cells with homologous antiserum. Trypsin did not affect either SA-cells or uncoupled cells. The trypsin diluent was also devoid of activity. The concentration of trypsin employed was sufficiently greater than that expected as a contaminant, to rule out the possibility of trypsin-like activity in the DNA-ase preparation.

One conclusion to be considered from above data, is that DNA functions in a manner analogous to haptens, *i.e.*, the DNA confers specificity, but it must be coupled with a carrier

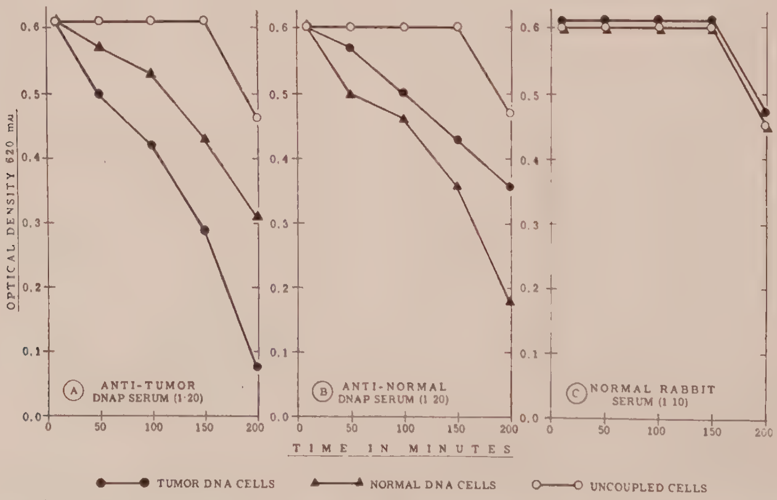

FIG. 1. Settling rate of coupled and uncoupled formalinized cells in presence of rabbit antiserums and normal rabbit serum.

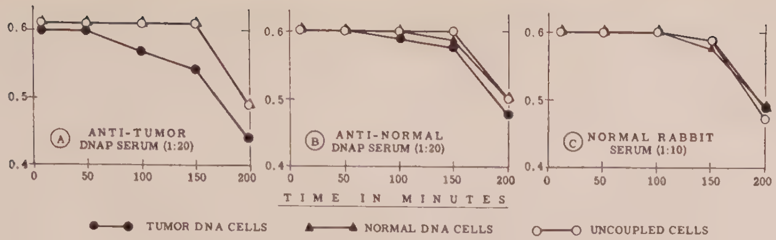

FIG. 2. Effect of DNA-ase upon settling rate of coupled and uncoupled cells in presence of rabbit antiserums and normal rabbit serum.

substance to stimulate antibody formation or to be detected by precipitin or agglutination reaction.

However, it must be admitted that it is difficult at present to understand why a hapten of the complexity of DNA would not precipitate with antiserum. This observation suggests that there might be an insufficient number of specific reactive sites per DNA molecule or that non-specific factors are involved, as observed by Ingraham(5) with respect to non-agglutination of formalin-treated red cells.

Summary. Formalinized erythrocytes exposed to purified DNA under the proper conditions can then be specifically agglutinated with antiserum, which has been prepared against the DNA-protein complex. Inhibition of the reaction by homologous DNA is strong evidence that agglutination is an antigen-antibody reaction and not merely a non-specific agglutination. The removal of this reactivity by desoxyribonuclease lends further support to the belief that DNA is functioning as the hapten. Trypsin does not alter the agglutination reaction.

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Growth-Promoting Properties of Pyruvate, Oxalacetate, and α-Ketoglutarate For Isolated Walker Carcinosarcoma 256 Cells. (24025)

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Puck and his associates devised procedures for growing isolated human cells in tissue culture(1,2). The medium consisted of whole serum, amino acids, vitamins, and balanced salt solution. Media containing dialyzed serum as replacement for whole serum did not sustain growth of single cells of the S3 colony strain of HeLa cells. Supplementation of the medium with cholesterol and a variety of coenzymes, however, enabled the cells to grow (3).

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The present communication concerns rapid growth of isolated Walker carcinosarcoma 256 cells induced by pyruvate, oxalacetate, and α-ketoglutarate in media containing dialyzed serum.

Methods. The basal medium (Table I) contained amino acids previously found essential or stimulatory for massive cultures of Walker tumor as well as 5 nonessential amino acids(4). Bovine serum was dialyzed against 2 changes of 100 volumes of Earle's solution at 6°C each for 12-24 hours on a shaker. Earle's solution used was modified to contain 3 g glucose/l, and an adjusted NaCl level to compensate for osmotic changes. Cells for inocula were grown from freshly excised tumor tissues in medium similar to the basal medium except that it contained 10% whole bovine serum. The inoculum was prepared by removing the used medium and separating cells by treatment with 0.05% trypsin (Bacto-Difco 1:250) 5 to 10 minutes at room temperature with occasional gentle agitation. Petri dishes were prepared in duplicate to contain 100 cells and graduated quantities of test compounds in 5 ml of basal medium. The dishes were incubated at 37°C for 7 days in air mixed with 7-9% CO_2 to maintain pH at approximately 7.2. For some of the work, cells were prepared by chopping fragments of excised tumor immersed in basal medium until a milky suspension of cells formed. This suspension was filtered through a pledget of glass wool. The inoculum obtained was utilized in the same manner as trypsinized cells.

TABLE I. Basal Medium for Study of Growth-Promoting Effects of Intermediary Metabolites and Other Factors upon Isolated Walker Carcinosarcoma 256 Cells.

Constituent	Conc.[*]
Amino acids	mM/l
L-Arginine · HCl	.2
L-Histidine · HCl · H_2O	.1
L-Lysine · HCl	.2
Glycine	.1
DL-Alanine	.3
DL-Serine	.5
DL-Threonine	.3
DL-Valine	.3
L-Leucine	.3
L-Isoleucine	.3
L-Aspartic acid	.15
L-Glutamic acid	.15
L-Asparagine · H_2O	.3
L-Glutamine	1.5
DL-Phenylalanine	.2
L-Tyrosine	.1
DL-Tryptophan	.03
L-Proline	.15
Hydroxy-L-proline	.15
L-Cysteine · HCl · H_2O	.2
DL-Methionine	.2
Vitamins	μg/ml
Thiamine · HCl	.2
Riboflavin	.2
p-Aminobenzoic acid	1
Pyridoxine · HCl	.5
Pyridoxal · HCl	.5
Nicotinic acid	.5
Nicotinamide	.5
Calcium pantothenate	.2
Biotin	.2
Folic acid	.2
Vitamin B_{12}	.000075
Choline chloride	5.0
Inositol	9.0
Ascorbic acid	.5
Glutathione	.5
Salts	g/l
NaCl	6.46
KCl	.4
$MgSO_4 \cdot 7H_2O$	.2
$NaH_2PO_4 \cdot H_2O$	.14
$NaHCO_3$	2.20
$CaCl_2$	.2
Misc.	
D-Glucose	4.0 g/l
Phenol red	2.5 μg/ml
Potassium penicillin G	50 "
Streptomycin sulfate	50 "
Dialyzed bovine serum[†]	15% (vol)

[*] Concentration in final culture medium.

[†] Dialyzed 2 times for 12-24 hr against 100 vol of Earle's sol.

Results. In early studies the cells produced colonies with 50-100% plating efficiency in basal medium containing whole bovine serum. Use of dialyzed serum for determination of amino acid requirements of isolated cells, however, resulted in formation of few or no colonies. Addition of coenzyme A, co-carboxylase, diphosphopyridine nucleotide, flavinadenine dinucleotide, and cholesterol as suggested by Sato *et al.*(3) did not improve the response of the cells. Intermediary metabolites, pyruvate,[†] oxalacetate,[‡] or α-ketoglutarate,[§] proved quite efficacious in promoting high plating efficiency and rapid cell division (Table II). The effects were detectable at

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[†] Nutritional Biochemicals Corp., and Distillation Products Industries.

[‡] Mann Research Laboratories, Inc.

[§] H. M. Chemical Co.

TABLE II. Response of Isolated Walker Carcinosarcoma 256 Cells to Selected α-Keto Acids.*

Metabolite, mM/l	Pyruvate		Oxalacetate		α-Ketoglutarate	
	Colonies	Cells/colony†	Colonies	Cells/colony†	Colonies	Cells/colony†
.0	0	38– 60	4	11– 40	0	0
.01	11	43– 65	16	31– 119	17	14– 22
.02	30	98– 171	9	118– 221	36	67–304
.05	81	660– 728	90	804–1048	87	400–468
.1	85	660–1060	89	529–1140	79	572–772
.2	89	888–1148	94	1412–1484	97	616–748
.5	98	1243–1398	90	1192–1664	99	988–996

* Initial inoculum was 100 cells/petri dish; cultures were incubated 7 days at 37°C.

† Actual cell counts in 2 colonies representative of estimated 60% of colonies.

0.01 mM/l and almost optimal at 0.05 mM/l. In presence of these intermediates, plating efficiency approached 100%. The number of colonies in duplicate petri dishes usually agreed within less than 10%. The colony size easily attained 100 cells in 7-day incubation periods. Colony size did vary possibly because of the heterogeneous cell population, occasional colony formation from more than a single cell, or local variation of conditions in culture vessels. Small differences in generation times could result in appreciably different colony size after 10 generations which occurred in 7 days. A measure of cellular growth, however, was obtained by counting cells in colonies representative of 60% of the colonies.

Intermediary metabolites displayed similar activity, and this activity was the same when equivalent molar amounts of all combinations of these compounds were tested. Succinate, fumarate, citrate, iso-citrate, and *cis*-aconitate, and lactate were inactive. The related L-alanine, L-aspartic acid, L-glutamic acid, L-glutamine, and L-asparagine were already included in the basal medium which supported little or no growth; and nonessential amino acids—L-alanine, L-aspartic acid, L-glutamic acid, L-proline, and L-hydroxyproline—in quantities as high as 10 mM/l did not produce the stimulatory effects noted here. Furthermore, growth-promoting properties of active metabolites were not diminished by omission of the nonessential amino acids, ascorbic acid, and glutathione.

Cells prepared directly from freshly excised tumor responded to intermediary metabolites as did the trypsinized cells. These fresh cells were not used for routine studies because debris and proportion of damaged cells rendered initial enumeration less reliable.

One effect of dialysis of serum on clonal growth of isolated Walker cells was the removal of pyruvate, oxalacetate, and α-ketoglutarate from the serum. These compounds are reported present in blood at approximately the levels (0.1 to 0.2 mM/l)(5) which supported optimal growth of cells. Massive cultures of the Walker tumor did not require these compounds and media employed by Sato *et al.*(3), Eagle(6), McQuilkin *et al.*(7), and Healy *et al.*(8) were devoid of these intermediary metabolites. Fischer's Medium 605(9), however, did contain them. The role of these metabolites in survival of isolated Walker cells is, at present, unknown; but these results suggest many interesting facets of tumor cell metabolism. For example, α-keto acids are well known for their participation in transamination processes in addition to their role in energy production, and these reactions may assume a more important role under conditions of clonal growth. The inclusion of coenzymes in the present medium could not be used in lieu of pyruvate, α-ketoglutarate, and oxalacetate.

Although future studies of exhaustively dialyzed serum or serum fractions may reveal requirements for further small molecular compounds, the present media containing twice dialyzed bovine serum and pyruvate, oxalacetate, or α-ketoglutarate is practical and effective for investigation of nutritional requirements of isolated Walker tumor cells and its clonal strains. Our experiments utilizing such media invariably led to a high plating efficiency and rapid growth of isolated Walker carcinosarcoma 256 cells.

Summary. When Walker carcinosarcoma 256 cells were grown under clonal growth technics, 50-100% plating efficiency was noted in a medium containing whole bovine serum. When dialyzed serum was used in the medium, few or no colonies were formed. Addition of pyruvate, α-ketoglutarate, and/or oxalacetate to the substrate resulted in plating efficiencies of 90-100%. Activity of these 3 metabolites was the same irrespective of addition of individual compounds or combinations thereof. Other Kreb's acids and metabolites tested failed to promote survival and growth of isolated Walker cells.

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Observations on Arteriovenous Communications in Lungs of Dogs.[*] (24026)

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The presence of precapillary arteriovenous communications in mammalian lungs has been one of the interesting questions of pulmonary vascular physiology. The study of Prinzmetal *et al.*(1) is often cited as evidence for these vascular communications. However, other workers(*2*,3) have suggested that precapillary shunts in the lungs are either very small or are possibly not extant at all. Because of the importance of these vascular passages, if present, in the economy of the pulmonary circulation, the following investigation was carried out.

Method. Mongrel dogs (8-14 kg) were anesthetized with sodium pentobarbital, and conventional Starling heart-lung preparations were set up. The arterial cannula was provided with a large side arm so that left ventricular output could be diverted to collecting vessels. Blood was heparinized. Pulmonary arterial pressure was recorded with a mercury manometer connected into the right upper lobar arterial branch. Cardiac output was adjusted to 400-500 ml/minute. Peripheral resistance was 80-90 mm Hg. Mean arterial pressure was usually 10-15 mm Hg. The preparations functioned without visible dilatation of the hearts. Lucite microspheres[‡] in 2 g quantities were suspended in dog plasma colored with Evans blue dye and were rapidly introduced into the right atrium through stoppered venous cannula. Spheres of 75 μ and 250-300 μ were assorted by screening and agitation and the 2 sizes of spheres were used in all experiments since this quantity produced moderate rises in pulmonary arterial pressure. Two grams of 75 μ spheres comprise 1 million particles by count and 2 g of 250-300 μ particles number 500,000. After introduction of the plasma-sphere suspension, as the dye appeared in the arterial cannula, the cardiac output was diverted for 45 seconds through a stainless steel screen (325 mesh) to sieve out spheres which passed through the

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[†] Nat. Heart Inst. research fellow.

[‡] Lucite microspheres were kindly furnished by E. I. du Pont de Nemours & Co., Wilmington, Del.

TABLE I. Results of Bead Injections in Conventional Heart-Lung Preparations.

Bead wt, g	Mean pulmonary art. press. mm Hg	Mean arterial blood pressure	Dog wt, kg	Beads recovered pulm. ven. blood	Size, μ
2	20 to 40	Decreased	14.5	0	75
2	20 50	"	11.3	+	"
1.75	5 40	"	10.2	+	"
1.75	10 45	"	10.4	+	"
1.5	10 30	"	12.7	+	"
2.5	20 45	"	10.9	0	300
1.9	5 20	"	14.5	0	"
2.5	20 45	"	8.0	0	"
2	20 40	"	9.0	0	"

lung vasculature. The atrial pressure was maintained at 15 cm of water by the addition of blood or saline to the venous reservoir. The screened spheres were identified by microscopic examination of the mesh.

Results are shown in Table I. In 4 out of 5 preparations in which beads of 75-80 μ were allowed to embolize the lungs, spheres were recovered from the pulmonary venous blood on the sieve. The retrieved particles represented about 0.5% of the total quantity introduced. On the other hand, in heart-lung preparations embolized with spheres of 250-300 μ, no particles were found on the collecting sieve. The consistent decline of systemic arterial pressure and elevation of pulmonary arterial tension after embolic assaults indicated that beads had reached small pulmonary arteries. After death of the preparations embolized with large spheres, no particles were found in the pulmonary veins, left cardiac chambers nor in the arterial cannula or tubing; however, spheres were readily identified throughout the pulmonary arteries.

Comment. From our data, there is evidence for the presence of pulmonary arteriovenous shunts in dogs, in that spheres of 75-80 μ appeared to pass through the lung vasculature under pressures engendered by the right ventricle. These data imply that the largest pulmonary arteriovenous connections are between 75 and 250 μ diameter. Since these heart-lung preparations are denervated it is possible that dilatation of any arteriovenous anastomoses had occurred maximally, thereby facilitating passage of the spheres.

Demonstrations of pulmonary precapillary AV shunts by embolic passage in our experiments and in those of others(1,5-8) are predicated on the assumption that pulmonary capillaries are in the order of 7 μ diameter(4), and that spheres of larger size can traverse the lung vasculature only through larger arteriovenous connections circumventing the capillary beds. Tobin and Zariquiey(5,6) injected suspensions of microspheres into pulmonary arteries at pressures up to 300 mm Hg, and identified particles of 500 μ in pulmonary venous blood. They suggested that the largest AV shunts were in lobar subdivisions of bronchopulmonary segments. Sersi and Bucher (7) concluded that 14.7% to 34.5% of radioactive particles (25-30 μ size) passed through AV communications to the pulmonary veins. Recently Niden and Aviado(2) noted the transit of spheres (60-420 μ) through the lung vasculature of dogs. Passage was increased by raising pulmonary perfusion pressure, or by the production of anoxia.

Experimental evidence of pulmonary AV shunts has not been unanimously accepted. Gordon, Flasher and Drury(9), using surface tension technics, noted that the largest AV connections in dog and rabbit lungs were about of 16 μ diameter. Knisley, Satterwhite and Wallace(10) reported their inability to recover spheres of 10 to 250 μ after injection at various sites into the veins, although in 18 of 36 animals small numbers of beads of 10-130 μ did traverse the lung vessels. They doubted the presence of precapillary anastomoses in normal lungs. They also suggested that recovery of beads in other experiments was due to escape of the particles into the vena cava and to the liver, or to excessive perfusion pressure forcing beads through capillaries or Thebesian veins thereby simulating AV transfers that may not exist.

Use of the heart-lung preparation precludes passage of microspheres into channels other than the pulmonary artery, and assures the propulsion of particles into the vasculature under physiological pressures. The recovery of spheres of 75-80 μ in the pulmonary venous blood leaves little doubt of the possibility of arteriovenous anastomoses in the pulmonary vasculature. Presumably these anastomotic connections function under conditions of increased pulmonary arterial pressure (such as occur in pulmonary embolism or heart failure) to spare the alveolar capillaries from overload. Other implied functions of these pulmonary AV shunts, as well as discrepant information as to their size, require further investigation.

Summary. Lucite spheres of 75-80 μ introduced into the pulmonary arteries of heart-lung preparations under physiologic pressures will pass from pulmonary arteries to veins. In contrast, microspheres of 300 μ size did not pass through the pulmonary vasculature.

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Influence of Varying Proportions in Combinations of Estrone, Estradiol and Estriol on Biological Response. (24027)

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Advances in analytical procedures, such as countercurrent distribution, as well as column and paper chromatography, have made it possible to identify and isolate estrogenic metabolites in relatively small (24-48 hour) collections of urine and in turn have stimulated much research. As a result, the interaction of these metabolites from a biological standpoint becomes even more intriguing. Hisaw and coworkers(1) and Wicks and Segal(2) stressed time and dose relationships. The concept of "impeded estrogens" was presented by Huggins and Jensen(3) and Edgren(4,5). Our interest in biological effectiveness of combinations of steroid hormones stems from 1948 when we reported results of androgenic mixtures on biological response(6). The present study was initiated in 1949.† Our aim was to see by what combination a synergistic effect could be realized or, if there was an inhibitory effect, at what level and in what combination it would be observed. From such data it may be possible to determine the type of biological activity that can be correlated with chemical assays, which determine presence and quantity of each estrogen in urine. A similar concept was postulated by Brown(7). Reports dealing with interaction of estrogen mixtures indicate that some workers employed either spayed mature(1) or hypophysectomized immature rats(3). Others employed intact immature mice(2,4,5). Other variables consisted of dose ranges, combination of estrogens and method of administering the mixtures.

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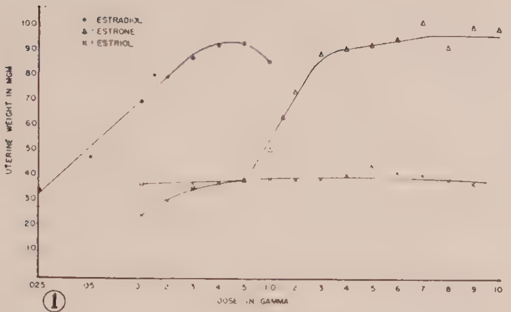

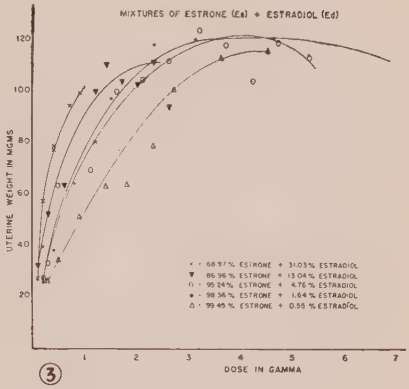

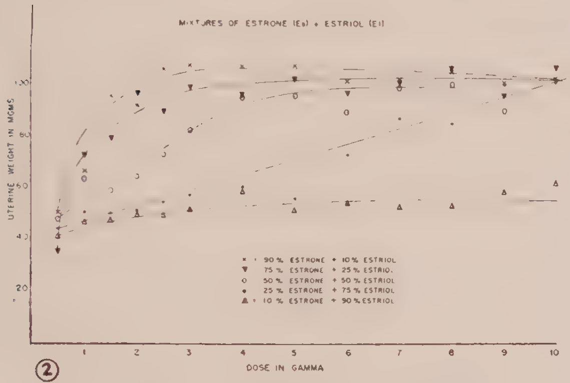

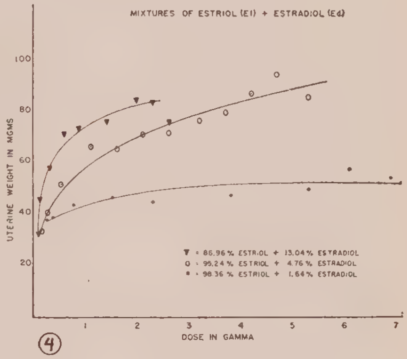

FIG. 1. Basic dose response curves established for estradiol 17 β, estrone and estriol 16 α, 17 β by uterine wt method in immature rats.

FIG. 2. Synergism is observed in the first 3 mixtures of the combination of the relatively biologically inactive estriol 16 α, 17 β with estrone. There is no inhibition of estrone by estriol 16 α, 17 β in any of the dose ranges in these mixtures.

FIG. 3. Inhibition appears in all combinations in the lowest dose range. Synergism occurs, depending upon proportions present in last 2 mixtures.

FIG. 4. Synergism is present in the very low dose range. Inhibition occurs only in the last mixture when estriol 16 α, 17 β portion was greater than 1.5 γ.

These variables tend to militate against valid comparisons of results obtained by different workers. We felt that simplification, by employing estrone as a standard for comparison with estradiol and with estriol in varying combination of mixtures, would be desirable. We chose the immature rat as test animal to eliminate response of target organ to preliminary stimulation or regression.

Methods and material. 2414 twenty-four day old immature rats of the Sherman strain were used. We employed 650 rats to establish basic dose response curves for the 3 estrogens studied (Fig. 1). 1613 animals were used to obtain data on mixtures that were studied and an additional 144 control animals were employed during the testing procedures. The rats were 24 days of age at time of first injection. Various doses of estriol, estradiol, and of estrone and combinations of these were dissolved in 0.6 ml of sesame oil and injected subcutaneously into the dorsum of each rat for 3 successive days. Rats were sacrificed on morning of fourth day. Body weights were recorded and the uteri removed and weighed after expressing the fluid. The ratio of body weight to uterine weight was calculated for a number of animals and was relatively constant. Therefore, only uterine weights were used to plot the curves. First we sought to establish the basic dose-response curves for estrone, estradiol 17β and estriol 16α, 17β (Fig. 1) using the uterine weight method. Relative potency of estriol and of estradiol with estrone was then compared by determining equation of the line using the method of "least squares" and comparing the slopes. Previously(6) we combined biologically active androsterone with the much less active dehydroisoandrosterone, and achieved striking biological enhancement. We decided to use the same mixtures if possible for combinations of estrone and estriol. The mixtures (Fig. 2) were therefore made on weight basis and total dose range for each mixture was 0.5 to 10.0 γ. The mixtures were as follows: 90% estrone - 10 estriol, 75 - 25, 50 - 50, 25 - 75, 10 - 90%. In making mixtures wherein the very biologically active estradiol was to be combined with estrone in one case (Fig. 3) and with estriol in the other (Fig. 4), it was necessary to consider the fact that estradiol was approximately 20 times as active biologically as estrone. We wished, therefore, to keep the estradiol content in mixtures containing it on an equivalent biological basis with those set up for estrone and estriol. This was done as follows: *Mixture I* was made by combining 1 part of estradiol with 180 parts of estrone, which results in a mixture containing 99.45% by weight of estrone and 0.55% by weight of estradiol. On a biological basis, however, estradiol being 20 times as active as estrone, the above becomes biologically equivalent to 20 parts of estradiol combined with 180 parts of estrone or in a ratio, 1 part of estradiol to 9 parts of estrone. The other mixtures were made up as follows: *Mixture II*: 60 parts estrone + 1 part estradiol or 98.36% + 1.64%. Biologically: 60 parts estrone + 20 parts estradiol. *Mixture III*: 20 parts estrone + 1 part estradiol or 95.24% + 4.76%. Biologically: 20 parts estrone + 20 parts estradiol. *Mixture IV*: 20 parts estrone + 3 parts estradiol or 86.96% + 13.04%. Biologically: 20 parts estrone + 60 parts estradiol. *Mixture V*: 20 parts estrone + 9 parts estradiol or 68.97% + 31.03%. Biologically: 20 parts estrone + 180 parts estradiol. The mixtures for estradiol and estriol were made in exactly the same manner as the above. Estradiol combined in this manner results in dosages that in all cases are lower than or approximately the dose level required to achieve its maximum response.

Results. Reference to the basic dose response curve (Fig. 1) showed that estriol was the least active of the estrogens in promoting uterine growth and that estradiol was the most potent. A linear response could be secured for estrone up to a 3γ dose, for estradiol up to 0.15γ dose, and for estriol up to a 0.4γ dose. Beyond these doses there is a leveling off of biological response. The equations derived from the dose response data for each of the 3 estrogens in their respective dose ranges are as follows:

Estrone $.1\gamma$ to 3.0γ : $y = 23.3\,x + 29.0$
Estriol $.1\gamma$ to $.4\gamma$: $y = 42.3\,x + 21.9$
Estradiol $.02\gamma$ to $.15\gamma$: $y = 404.1\,x + 24.8$

By comparing the slopes of these equations it can be seen that estradiol is 17.4 times as effective as estrone. This value is not too far removed from the 20:1 ratio observed by other workers (Lauson, 8). We found, as did Lauson(8) that estriol was *more* potent than estrone *in the very low dose range* (*below 0.4γ*). Our experiments showed the significant fact that estriol is 1.8 times as potent as estrone at this level (cf. Discussion).

Comparisons within the 3 dose response curves hold only for the limits of effectiveness of the compared individual estrogens. It does not follow that 1γ of estradiol when injected into an animal will be equivalent in effect to 17.4γ of estrone, since the peak of biological response as far as estrone is concerned is reached at 4γ. Any further increase in dosage results in a leveling off or plateau as shown in the curves. It will be shown later that it is only when mixtures of the various estrogens are used that it is possible to exceed this peak.

On the basis of these responses we were interested in what effect the combination of a fairly biologically inert estrogen, such as estriol with estrone alone and with estradiol alone would have. The second part of the experimental study deals with these mixtures and the results that were obtained.

The results of the study of these varying proportions of mixtures of estrone + estradiol; estrone + estriol; and of estriol + estradiol show that each combination exhibits behavior peculiar to that pair of estrogens.

The results obtained with each combination are therefore described separately as follows:

Estrone + estriol (Fig. 2). The combination of the relatively inactive estriol with estrone resulted in the most striking synergistic effects. In the mixtures of 90% estrone with 10% estriol, 75% - 25%, and 50% - 50% synergism was observed in the total dose range from 1 to 5γ. Beyond the total of 5γ no synergism was observed but neither was there an inhibitory influence of estriol on estrone. Increments of estriol from 0.05γ to 5.00γ when mixed with estrone in the above mentioned proportions produced synergism. In the mixture of 25% estrone with 75% estriol synergism was attained only in the highest dose range, and not at all in the mixture of 10% estrone with 90% estriol. It is significant also that estriol did not inhibit the estrone activity *per se* in any of the mixtures. Reference to the curves for the respective mixtures of the estrogens and to the basic dose response curves was made in order to determine whether or not synergism[‡] had occurred. This determination was made as follows: The response[§] to a dose for a given mixture was read from the curve of that mixture. This value was then compared to the sum of the responses for the constituents of this dose as determined from the individual basic dose response curves. This procedure was used to interpret all other combinations of the estrogens.

Estrone + estradiol (Fig. 3). Mixtures of varying proportions of estrone with estradiol (Fig. 3) revealed a distinctly different pattern from those when the relatively inactive estriol was combined with either one of these more potent estrogens (Fig. 2-4). In the very low dose range for each mixture the combination of estrone and estradiol resulted in an inhibitory effect on uterine stimulation. This was almost akin to antagonism. As the dose was increased, synergism was produced in a varying degree dependent upon the proportion in which the 2 estrogens were combined. In the first mixture, wherein 99.45% by weight of estrone was combined with 0.55% by weight of estradiol, synergism was achieved in the total dose range from 0.905 to 4.525γ. In the mixture consisting of 98.36% by weight of estrone and 1.64% by weight of estradiol, synergism was achieved in the total dose range from 0.381 to 4.58γ. A tenuous enhancement was achieved for a very narrow dose range in the next 2 mixtures. It is interesting to note that the peak of biological responsiveness for these mixtures was at a higher uterine weight level than the peak for either estrone or estradiol alone. We can summarize our observations as to the effect of mixtures of estrone and estradiol on the immature rat uteri as follows: There is a dose range which results in an inhibition of uterine growth followed by a dose range which results in stimulation of this growth to a point of synergism and a dose range beyond which the animal cannot respond—a so-called point of non-responsiveness.

Estriol + estradiol (Fig. 4). The results obtained with mixtures of estriol and estradiol showed that here again it was possible to achieve synergism but only in the very low dose range of the 3 mixtures in this combination. In the mixture consisting of 98.36% estriol and 1.64% estradiol, we found evidence for inhibition of estradiol by estriol when the estriol portion of the total dose was greater than 1.5γ. In the other 2 mixtures, however, estriol did not inhibit the effect of estradiol even after its portion of the total dose had gone over 1.5γ. It is realized that a dose of 1.5γ of estriol is far beyond the maximum biological response dosage for estriol but this was the only region where inhibition was manifest.

Discussion. The synergistic effect which was obtained with mixtures in the low dose range was a fairly consistent observation in all of the combinations that were studied with the exception of the estrone + estradiol mixture. In this combination an inhibitory effect on uterine stimulation almost akin to antagonism was observed in the very low dose range.

[‡] The term synergism is employed only when uterine response to the dose of the estrogenic mixtures is greater than or equal to the sum of the individual uterine responses for each constituent of the mixture.

[§] The term response is used for net uterine weight, i.e. data corrected for control weight.

In specific instances there is an enhancing influence by the almost biologically inactive estriol. This points up an important consideration in the investigation of the influence of minute amounts (up to 0.4γ) of presumably inactive hormones in maintenance of hormonal balance. The most striking effect of this study of mixtures is the behavior of estrone + estriol combinations where synergism was observed up to the 5γ dose level for 3 out of 5 mixtures. It was likewise significant that estriol did not inhibit estrone activity in any of the mixtures. The potentiation resulting from mixtures is unexplained. Whether it occurs at the target tissue level or as the result of chemical changes of the compounds by the other tissues of the test animal is to be pursued. It is conceivable, however, that with the combination of estrone and estradiol, a mass action effect takes place *in vivo*. Presumably the estrone retards the conversion of the estradiol to estrone and thus increases the net estradiol actually present in the mixture at target organ level to create the effect we observed as synergism.

The synergistic effects observed with estrone + estriol may be due to a block of an active degradation site of the estrone to estriol thus increasing the estrone-estradiol interchange. The possible influence of the presence of estriol during the presumed conversion of estrone to estradiol is to be developed. On the other hand, when combinations of estrone and estriol reach a dose level beyond the peak of response for estrone alone, there is a decrease in response which may be accounted for by a biological reaction which disturbs hormonal equilibrium or, it may be accounted for by a lessening of activity due to a competitive action by both of these estrogens for the active sites of the target organ.

The explanation for the inhibition of estradiol by estriol in the 98.36% estriol + 1.64% estradiol may also be due to competitive action by these 2 estrogens for the target organ. In this mixture the amount of estriol present is considerably beyond the limit of its biological effectiveness. In competition for active sites in the target organ it may overwhelm the amount of estradiol present. In the other mixtures in this series the amount of estradiol is of such proportion that as it approaches its peak of response it is able to counteract the effect of the estriol at the tissue level.

An explanation of the synergism exhibited by estradiol and estriol in the low dose range may be on the same basis as the suggested mechanism at work when estrone was combined with estriol. Or, it could be speculated that at the low dose level there is no competition for the target organ and that a summation of effect is being attained.

Summary. Basic dose response curves for estrone, estradiol, and estriol were established using uterine weight method in immature female rats of the Sherman strain. Combinations of estrone + estriol; estrone + estradiol; and estradiol + estriol were set up to study their effect on stimulation of the immature rat uterus. The interpretations were based on the data of dose response curves. A synergistic effect was observed in all of the mixtures studied in the low dose level except with the mixture of estrone + estradiol, which showed it in the higher dose levels. A combination of estrone + estriol resulted in synergism being observed for 3 out of the 5 mixtures studied up to a 5γ dose level. Estriol did not inhibit estrone activity in any of the mixtures. In the study of the 3 estradiol + estriol mixtures there was evidence of inhibition of estradiol by estriol in only one of the mixtures. The manner of action of these combinations of estrogens has been postulated.

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In vitro Effect of Surface Active Agents on Human Serum Lipoprotein and Protein Patterns.* (24028)

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Several authors have described changes in electrophoretic migration rates of serum lipoproteins under various conditions. Sachs and Danielson(1) recently reported that incubation of soy bean phosphatides with human serum produced increased migration velocity of serum lipoproteins on paper electrophoresis. Laurell(2) and Gordon(3) previously reported that incubation of serum with fatty acids produced a similar effect on migration of serum lipoproteins. Injection of heparin into hyperlipemic subjects also causes increased velocity of migration of lipoproteins when analyzed by electrophoresis technic(4). However, this effect occurs only *in vivo*. In a study of mechanisms involved in producing these changes several surface active agents of different characteristics have been investigated to determine their effects on serum lipoprotein and protein patterns.

Materials and methods. The following surface active agents were incubated with normal and lipemic human serum: a) sodium lauryl sulfate; b) commercial aryl-alkyl sulfonated anionic detergent, Lakeseal;‡ c) non-ionic polyoxyethylene sorbitan detergent, Tween 80; d) cationic detergent, benzalkonium chloride; and e) equal mixture of Tween 80 and benzalkonium chloride. Corn oil phosphatide preparation,§ containing lecithin, cephalin, and inositol phosphatides was also incubated with serum. Five to 20 mg of each of above agents were added to 2 ml serum aliquots, and equal aliquots without added agents served as controls. Sera were incubated at 37°C for 18 hours. After incubation samples were subjected to paper electrophoresis and to ultracentrifugal studies. Electrophoresis of samples was performed on Spinco Model R electrophoresis system in barbital buffer of pH 8.6 and 0.75 ionic strength. The apparatus was operated at 5 milliamperes for 16 hr. Serum aliquots of .01 ml were used for protein staining, and .02 ml aliquots were used for lipid staining. Protein was stained with bromphenol blue, and lipid with oil red 0 according to modified method of Durrum(5). Ultracentrifugation studies were performed on Spinco Model E analytical ultracentrifuge by the method of Gofman and associates(6,7). The above agents were incubated with normal serum and with serum lipoproteins obtained by preparative ultracentrifugation of serum at 30,000 rpm for 13 hr at specific gravity 1.0635. In the latter case serum lipoproteins were concentrated 3-fold before incubation with the agents.

Results. Table I shows effects of various surface active agents on migration rates of serum lipid components. Anionic surface active agents, sodium lauryl sulfate and Lakeseal, when incubated with serum increased migration velocity towards the anode of both alpha and beta lipid fractions at pH 8.6 when compared to control patterns. Incubation of

TABLE I. Effects of Various Surface Active Agents on Migration Rates of Serum Lipoproteins *In Vitro.*

Sample	Mean distance moved from origin at 5 milliamperes in 16 hr in mm	
	β-Lipoprotein	α-Lipoprotein
Sodium lauryl sulfate	+53 (+31)	+85 (+61)
Lakeseal	+49 (+30)	+84 (+61)
Tween 80	0 (+29)	0 (+61)
Benzalkonium chloride	+12 (+30)	+52 (+61)
Tween 80 + benzalkonium chloride	−36 (+31)	−25 (+75)
Corn phosphatide	+56 (+33)	+90 (+64)

Control values shown in parentheses.

(+) Plus sign designates movement from origin toward anode.

(−) Minus sign designates movement from origin toward cathode.

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† Research trainee, Public Health Service.

‡ Supplied by Finger Lakes Chemical Co.

§ Avitol supplied by Mr. M. Mattikow of Refining Unincorp., N. Y.

TABLE II. Effects of Surface Active Agents on Ultracentrifuge Patterns of Serum and Concentrated Lipoproteins.

Sample	Lipoprotein conc. S_f 0-12 (mg %)	Lipoprotein conc. S_f 12-400 (mg %)
Serum lipoproteins		
Lakeseal	175 (200)	138 (135)
Corn phosphatides	281 (287)	227 (77)
Sodium lauryl sulfate	8 (200)	95 (125)
Concentrated lipoproteins		
Lakeseal	244 (266)	243 (128)
Corn phosphatide	55 (155)	216 (128)
Tween 80*	218 (287)	55 (77)
Tween 80†	48 (287)	12 (77)
Benzalkonium chloride	71 (266)	236 (128)

Control values shown in parentheses.

* Incubation for 6 hr. † Incubation for 18 hr.

serum with non-ionic detergent, Tween 80, resulted in complete loss of mobility of all serum lipids toward either electrode. The cationic detergent, benzalkonium chloride, when incubated with serum decreased migration velocity of both fractions towards the anode. No differences were noted between effects of any of these agents on normal and hyperlipemic serum.

Incubation of serum with anionic agents resulted in formation of prealbumin protein component on the electrophoretic pattern. This effect was especially pronounced by sodium lauryl sulfate, which was accompanied by diminution of alpha$_1$, alpha$_2$ globulins and albumin components. None of the other surface active agents produced changes in protein distribution as detected on paper electrophoresis.

Table II records the effects of surface active agents on lipoprotein ultracentrifugal patterns. No constant relationship was found between effects of these agents on electrophoretic and ultracentrifugal patterns. Sodium lauryl sulfate and Tween 80 appear to increase the density of lipoproteins present, while the cationic agent, benzalkonium chloride and corn oil phosphatide decrease the lipoprotein densities. The anionic agent, Lakeseal, shows less pronounced changes on lipoprotein densities. Differences between control values represent usual variations found in lipoprotein concentrations of different sera.

Discussion. Results obtained after incubation of serum with surface active agents of different ionic charges offer a possible explanation for the mechanism of effects produced on lipoproteins by soy bean phosphatides, fatty acids and heparin injection. The results indicate that direction and rate of migration of serum lipids depend upon the charge present on ionic group of the active agent. This phenomenon might be readily explained by assuming that the surface active agent is bound by the lipoprotein molecule producing a net change in the charge of the molecule. Anionic agents would thus increase rate of migration of the lipid toward the anode as observed in this experiment. Depending upon degree of binding by lipoproteins the cationic surface agent would either decrease rate of migration towards the anode or cause direction of migration to be towards the cathode. Surrounding of lipoprotein molecules by the bound non-ionic agent, Tween 80, would decrease their migration in an electric field as evidenced experimentally by complete lack of mobility of serum lipid after incubation with Tween 80.

Friedman and associates(8) demonstrated that serum lipoproteins rapidly bind Tween 80 after intravenous injection. Injection of Tween 80 produces a marked hyperlipemic response by enhancing ability of lipoproteins to take up lipid from the liver. The results of our experiment indicate that Tween 80 enhances the binding of the cationic surface active agent, benzalkonium chloride, by lipoproteins. This increased binding of the cationic agent produces a marked reversal in migration direction of serum lipoproteins.

Binding of fatty acids by lipoprotein would result in an increased negative charge of the lipoprotein molecule and an increased migration rate of the molecule towards the anode. This mechanism was suggested by Gordon(3) to explain electrophoretic changes produced by interaction of sodium oleate and human serum. Similar effects were noted with soy bean phosphatides(1), and with a corn oil phosphatide used in this study. Sachs and Danielson(1) found the active component of soy bean phosphatide to reside in the alcohol-insoluble lipositol fraction. Although the complete chemical structure of lipositol is unknown, this phosphatide contains phosphoric acid esterified with inositol suggesting it to be an anionic agent. The effects of heparin on electrophoretic mobility of lipoproteins *in vivo* remain unexplained. However, the effects on both alpha and beta lipoprotein are qualitatively the same as those seen after incubation with anionic agents *in vitro*(4).

Lipoprotein densities studied ultracentrifugally are unrelated to ionic charges of surface active agents; however, density changes suggest binding of agents by lipoproteins.

The prealbumin fraction observed after incubation with anionic agents has also been reported after injection of heparin(9) and after incubation of serum with sodium oleate(3). Reasons for the marked difference between the effects of these agents on lipid and protein patterns are not apparent. If all lipids are bound to only a small portion of total serum, protein changes in the migration of this small portion might not be detected on paper electrophoresis.

Summary. The effects of incubation of serum with various surface active agents on serum lipids and protein patterns have been studied on electrophoresis and in the ultracentrifuge. Direction of migration of lipid in electrophoresis depends upon the charge of surface active agents. Anionic surface active agents increase migration rate of both alpha and beta lipoproteins toward the anode. Cationic agents either decrease migration rate of lipids toward the anode or cause them to move toward the cathode. Non-ionic agents cause complete loss of mobility of all lipids toward either electrode. These effects could be explained by assuming that surface active agents are bound to lipoproteins and contribute their charge in the migration of these lipoproteins. Effects of surface active agents on protein patterns are less pronounced. Surface active agents either increase or decrease density of serum lipoproteins as studied in the ultracentrifuge suggesting that these agents are bound to lipoproteins. However, prediction of effects of these agents on lipoprotein densities cannot be made from knowledge of the charge present on the agent.

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Measurement of Compounds of Vit. B_6 Group in Blood.* (24029)

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Using a spectrophotofluorometric technic (1), Duggan and Udenfriend have shown that the Vit. B_6 group could be detected in concentrations as low as 0.001 μg/ml(2). We confirmed these observations, and extended the use of this technic to demonstrate effects of hydrogen peroxide and ultraviolet irradiation on these fluorescent curves. Initial studies established optimal conditions for fluorescence of these compounds to be in 0.1 molar phosphate buffer of a pH 6.75. The ideal concentration for studying activity was found in the range of a 1 micromole/l. Table I outlines the fluorescence of a 1 micromolar stand-

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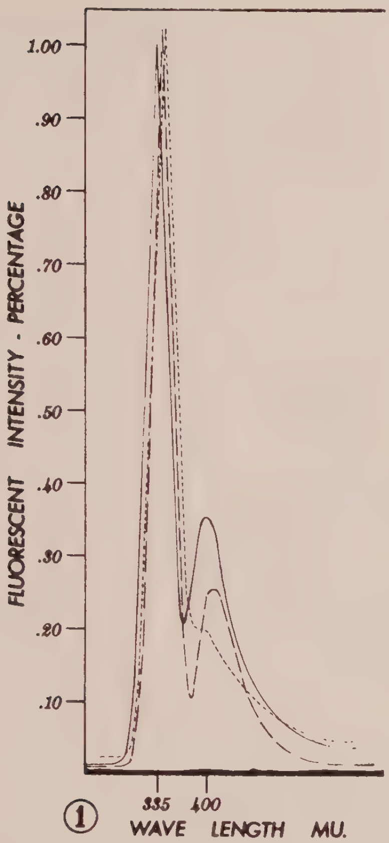
TABLE I. Fluorescent Characteristics of Compounds of Vit. B_6 Group under Selected Physical Chemical Conditions. The individual substances were of 1 micromolar concentration in phosphate buffer of pH 6.75. Photomultiplier readings at .001 sensitivity.

	Compounds of the vit. B_6 group		
Physical chemical reaction	Pyridoxine	Pyridoxamine	Pyridoxal
Activating, mμ	340	335	330
Fluorescent, mμ	400	400	385
Fluorescence, %	44	56	35
H_2O_2, 10 lambda			
3%	No effect	No effect	Partial destruction
30%	”	”	Complete
Ultraviolet			
1 min.	Partial destruction	Partial destruction	Partial destruction
5 ”	Complete	Complete	Complete

ard solutions of each of the free forms of Vit. B_6 to Xenon lamp activation; after exposure to 10 lambda quantities of hydrogen peroxide (3% and 30% concentration); and after exposure to ultraviolet irradiation (1 and 5 minute duration) using a quartz mercury vapor lamp at 3 cm. Hydrogen peroxide does not affect activity of pyridoxine or pyridoxamine. However, there is partial destruction of pyridoxal by a 3% solution and complete destruction by a 30% concentration. The effects of ultraviolet light were comparable in all 3 forms with partial destruction at 1 minute and progressive deterioration with time to complete disappearance of activity at 5 minutes. These experimental data suggested that if the minute quantities of Vit. B_6 present in blood could be isolated, these procedures would facilitate their identification and quantitation. Concern over the possibility of altering the fluorescent patterns led to exploration of a number of physical chemical approaches to the problem with ion exchange columns and extraction procedures. Of these, the most successful has been with acetone extraction of heparinized fresh human whole blood.

Method. A 1 ml sample of fresh whole blood was extracted with 25 ml of reagent grade acetone in an amber glass container. The mixture was shaken 30 minutes and then evaporated to dryness. The residue was taken up in 5 ml of 0.1 molar phosphate buffer of pH 6.75. The sample was cleared either by high speed centrifugation (20,000 rpm for 30 minutes) or by a Morton sintered glass filtration. A 1 ml aliquot was activated by the Xenon lamp at 335 mμ with simultaneous scanning showing a peak value for total fluorescence at 400 mμ. A 10 lambda quantity of a 30% solution of hydrogen peroxide was added to the sample to destroy pyridoxal activity, and the reading procedure repeated. The sample was then exposed to ultraviolet irradiation for 5 minutes to destroy pyridoxamine, and fluorescent output again determined. A comparison of readings obtained at these 3 peaks with known standards that had been similarly treated provided direct quantitative measurement of pyridoxal and pyridoxamine in the blood. The possibility of pyridoxine and pyridoxic acid being present in the sample was considered. However, studies with pyridoxic acid have shown it to be excited at an activating wave length of 330 mμ with a peak fluorescence at 430 mμ. Conceivably, traces of pyridoxine could be present, but well documented experiments with microbiological assay and ion exchange columns have repeatedly failed to demonstrate the presence of this form in blood(3,4).

Results. This procedure has permitted quantitative measurement of individual members of the Vit. B_6 group in standard solutions, and in whole blood *per se.* Furthermore, it has made possible recovery of known amounts of these compounds that have been added to whole blood. Blood samples were obtained on 3 occasions from a healthy male adult to establish the normal range of Vit. B_6 activity for the patient. Under fasting conditions, he was given 30 mg of pyridoxine hydrochloride orally every 4 hours for 6 doses total 180 mg. Blood samples were obtained at 4 and 24 hours after start of medication. Fig. 1 shows the fluorescence present in patient's blood prior to medication. Total emission is represented by the solid line. The effect of 30% H_2O_2 with destruction of pyridoxal and the residual pyridoxamine is shown by dashed line. Complete destruction of activity by ultraviolet irradiation is seen in the dotted line. Fig. 2 shows change in fluores-



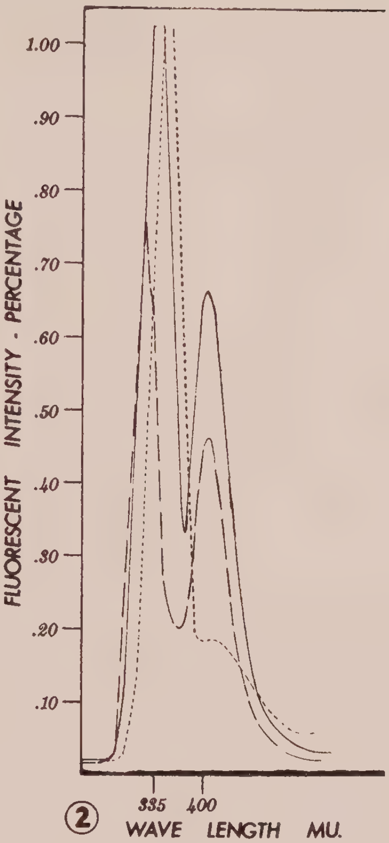


FIG. 1. Whole blood sample prior to ingestion of pyridoxine. Solid line (———) is total fluorescence transmission. Dashed line (– – –) represents fluorescence of pyridoxamine remaining after destruction of pyridoxal with 30% hydrogen peroxide. Dotted line (- - - -) is the blank produced by irradiation with ultraviolet light to destroy the pyridoxamine.

FIG. 2. Whole blood sample 4 hr after ingestion of 30 mg of pyridoxine. Solid line (———) shows increased total B_6 content. Dashed line (– – –) represents increased pyridoxamine following destruction of larger quantity of pyridoxal. Dotted line (- - - -) is residual blank after irradiation with ultraviolet light to destroy the pyridoxamine.

cence 4 hours after ingestion of 30 mg of pyridoxine. The total (solid line) is definitely increased. There are also higher values for pyridoxal and pyridoxamine as evidenced by height of curve following treatment with 30% H_2O_2 (dashed line). The effect of ultraviolet exposure (dotted line) provides a blank wave comparable to those seen before medication. These studies were done with samples that had been cleared with high speed centrifugation—leaving enough activating scatter to cause a minor effect on readings of fluorescent output. Ultrafiltration removed this material completely, and produced a zero blank.

It is of interest to note that the blood sample at 24 hours revealed curves and values slightly lower, but comparable to those for 4 hours. This would suggest that the blood transport of Vit. B_6 had reached a saturation point beyond which it could not carry additional quantities of the compounds. Concurrently, the patient's urinary excretion of pyridoxic acid increased markedly.

Summary. A study of fluorescent characteristics of pyridoxine, pyridoxal, and pyridoxamine under selected conditions has been presented. These have been applied to acetone extracts of standard solutions and whole blood with quantitative identification of all 3 components of the Vit. B_6 group. Data show that human blood contains pyridoxal and pyridoxamine. These two components can be made to increase to a limited maximal concentration of saturation upon the oral administration of pyridoxine.

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Decrease in Hexosamine Content of Epiphyseal Plates in Experimental Lathyrism.* (24030)

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Lathyrism is caused by ingestion of excessive amounts of sweet pea seeds (*Lathyrus odoratus*). The most characteristic lesions are deformities of bones, enlargement of epiphyseal plates, dissecting aneurysm of the aorta, and loss of weight. Research in this field has increased greatly since Ponseti *et al.* (1-2) showed the possible relations between lathyrism and many human bone diseases of unknown etiology. The earlier literature on lathyrism has been reviewed recently by Selye(3). The disease can be reproduced experimentally by injection of beta-aminopropionitrile, the active principle of sweet pea, or by aminoacetonitrile which is still more active in reproducing the bone lesions. The nature of metabolic block produced by aminonitriles remains to be elucidated. Since the most evident lesions appear in ground substance of the epiphyseal plates, some interference with mucopolysaccharide or collagen metabolism can be supposed. For this reason we studied the content of hexosamine, a component of mucopolysaccharides, and hydroxyproline, characteristic of collagen, in normal and lathyric epiphyseal plates.

Methods. Twenty rabbits, 7-10 days old, were used. Ten were injected with aminoacetonitrile sulfate,§ the others were controls. Each experimental animal received subcutaneously 10 mg of aminoacetonitrile sulfate neutralized with $NaHCO_3$, daily for 6 days. With this dosage the animals showed very severe skeletal lesions; the epiphyseal plates were very wide and distorted as described by Ponseti and Shepard(1). Longer treatment resulted in death of animals. After 6 days all rabbits were killed with ether; epiphyseal plates (about 200 mg each) were excised from proximal and distal ends of long bones (radius, ulna, tibia, femur) after removal of muscles and periosteum. The tissue was dried at 104°C to constant weight, then homogenized with 4N HCl in a Potter-Elvehjem homogenizer and refluxed at 100°C for 15 hours; a large excess of acid was used (500-600 times the quantity of tissue) to reduce humin formation. After hydrolysis, the acid solution was put in a vacuum desiccator with NaOH and $CaCl_2$ and the air evacuated. The dry residue was taken up in 25 ml H_2O. Hexosamine was determined by Schloss' method(4). Eastoe's method(5), using Dowex 50, was tried for a few hydrolysates, to separate glucosamine and galactosamine. Hydroxyproline was determined by the method of Neuman and Logan(6) according to suggestions of

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‡ Idem Chim. Generale.

§ The aminoacetonitrile sulfate has been kindly furnished by Dr. S. Wawzonek, Chem. Dept., State University of Ia.

TABLE I. Content of Hexosamine in Epiphyseal Plate of Lathyric and Normal Rabbits (mg/g Dry Wt).

	Normal, mg	Lathyric, mg
	89.4	57.9
	84.1	55.0
	104.1	55.6
	89.7	59.3
	85.3	53.8
	84.0	58.5
	84.7	58.4
	89.0	56.7
	89.2	57.2
	88.2	57.0
Avg	88.8	56.9
Decrease		36%

Martin and Axelrod(7). To obtain a clear solution, after adding paradimethylaminobenzaldehyde solution, we used 3 ml each of 5.3 N H_2SO_4 and 2.66% paradimethylaminobenzaldehyde instead of 4 ml of 4 N H_2SO_4 and 2 ml of 4% paradimethylaminobenzaldehyde, as called for in the Martin and Axelrod method.

Results. The results obtained in hexosamine analysis are reported in Table I. The values are expressed in mg of aminosugar for every g of dry tissue. The mean content of hexosamine in normal epiphyseal plate was 88.8 mg and in experimental tissue 56.9 mg, a decrease of 36%.

Some hydrolysates have been analyzed chromatographically on Dowex 50 to know the comparative content of galactosamine and glucosamine. As expected, the content of galactosamine was 9 times greater than that of glucosamine, which correlates with the fact that chondroitinsulfate is the principal mucopolysaccharide present in cartilaginous tissues.

Table II shows the content of hydroxyproline in normal and lathyric epiphyseal plates. Only small differences were found between experimental and normal cartilages.

Table II records also collagen values obtained by multiplying the hydroxyproline by 7.46. These values represent total collagen, including insoluble collagen and acid- and alkali-soluble pre-collagens that are probably present in pre-osseus cartilage.

Discussion. Owing to the decrease of galactosamine in epiphyseal plates of young rabbits injected with amino-acetonitrile, we could suppose that this compound interferes in some way with the metabolism of mucopolysaccharides. It is interesting that Dasler(8) was able to prevent characteristic lesions caused by aminonitriles by including l-glutamine in the diet of the animals. Glutamine is a precursor of hexosamine according to results obtained by Leloir and Cardini(9) in *Neurospora crassa.* Our research with homogenates of epiphyseal plates(10) showed formation of hexosamine from glutamine and glucose-6-phosphate. The enzyme essential to this synthesis, that may be called hexosamine synthetase, was found by us also in liver, callus of bone, rib cartilage and other tissues(11-12); its presence in liver was recently confirmed by Pogell(13,14).

The results reported here are not sufficient to prove a direct action of aminonitriles on mucopolysaccharide biosynthesis. Epiphyseal plates of lathyric animals are greatly widened and cell proliferation appears sharply increased(1); so a direct action of aminonitriles on protein synthesis, with a consequent change in ratio of mucopolysaccharides to proteins cannot be excluded. Study of this phase is planned.

Summary. The content of hexosamine and hydroxyproline in epiphyseal plates of lathyric and normal rabbits has been determined. A significant decrease (36%) of hexosamine was found in cartilage from experimental animals. Only small differences for hydroxyproline have been observed between experimental and control cartilage.

TABLE II. Content of Hydroxyproline in Epiphyseal Plate of Lathyric and Normal Rabbits (mg/g Dry Wt).

	Normal		Lathyric	
	Hydroxyproline	Collagen*	Hydroxyproline	Collagen*
	mg			
	19.6	146.2	19.8	147.7
	19.7	147.0	20.1	149.9
	18.4	137.3	19.9	148.4
	22.7	169.3	17.0	126.8
	20.7	154.4	19.8	147.7
	21.0	156.7		
Avg	20.3	151.8	19.3	144.1

* Hydroxyproline × 7.46.

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Effect of Tranquilizers and Other Agents on Sexual Cycle of Mice.* (24031)

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Some agents which act on the central nervous system have been shown to affect the reproductive system of animals. Daily subcutaneous doses of reserpine have been shown to interfere with estrus in mice(1) and to suppress ovulation and menstruation in monkeys (2). Chlorpromazine may delay ovulation and menstruation in women(3). Reserpine, chlorpromazine, pentobarbital, morphine, atropine, dibenamine and SKF 501, given as single doses prior to ovulation, can all block release of ovulating hormone in rats(4,5). Our work shows that some of these and other centrally acting agents interfere with estrus cycles in mice when given more or less continuously by drug-diet administration.[†]

Methods. Vaginal smears were obtained by saline lavage once daily from ZBC mice[‡] before, during and after treatment. Only animals with regular estrus cycles before therapy were used. Mice were housed in individual cages. Control diet was Purina laboratory chow. Drugs were given by drug-diet administration *ad libitum* with all drugs except alcohol, which was administered in drinking water *ad libitum*. Drugs, other than alcohol, were carefully powdered and thoroughly mixed with control food in the desired concentrations. The number of estrous cycles originating during treatment was compared with the number of cycles originating during the previous control period over the same number of days, usually 20. Thus mice served as their own controls. Cycles originating during the first 3 days of treatment were excluded.

Results. Table I gives results obtained with the various agents used. Reserpine (0.00075 and 0.001%), chlorpromazine HCl (0.3%), promethazine HCl (0.3%), phenaglycodol (1.0%), meprobamate (3.0%), phenobarbital (0.2%), ethyl alcohol (20%), atropine SO_4 (0.1 and 0.2%) and SKF 501 (1.0%)[§]

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[†] Author wishes to thank the following for generous supplies of drugs: Dr. Richard Tislow, for meprobamate (Equanil), promethazine hydrochloride (Phenergan) and promazine hydrochloride (Sparine); Dr. Robert Gaunt, for reserpine (Serpasil); Dr. Edwin J. Fellows, for chlorpromazine hydrochloride (Thorazine) and SKF 501; Dr. D. V. Hatton, for phenaglycodol (Ultran).

[‡] Mice were obtained from Dr. J. J. Bittner. ZBC mice are back cross $Z(C_3H)$ mice bred by mating AxZ F_1 hybrid females with Z males.

[§] SKF 501, or N-(9-fluorenyl)-N-ethyl-B-chloroethylamine hydrochloride, is an adrenolytic agent.

TABLE I. Effect of Drugs on Estrous Cycle of Mice.

Drug	Conc. in %*	No. of mice	% decrease in No. of estrous cycles†	No. mice with no estrus after day 3	% change in No. of cycles in post-treat. period against No. in pre-treat. period	Change in body wt in g due to therapy
Reserpine	.001	20	83.6 (20)	12	−21.9	−1.7
	.00075	9	74.1 (18)	5		− .9
	.0005	9	48.3 (20)	1		+ .1
Chlorpromazine‡	.3	10	70.0 (20)	4	+17.1	−1.4
	.2	10	46.6 (20)	0		− .8
Promazine§	.2	8	78.1 (20)	3	− 6.2	−5.6
	.1	10	48.8 (20)	1	0	−4.0
Promethazine	.4	10	100.0 (20)	10	−25.7	−6.0
	.3	10	71.1 (20)	5	0	+1.3
Phenaglycodol	1.0	10	100.0 (13)	10	−25.0	−1.5
	1.0	9	88.8 (14)	8	−23.5	−4.4
	.75	10	38.7 (17)	1	−25.0	−1.6
	.5	10	10.7 (15)	0	−14.3	+1.3
Meprobamate	3.0	10	88.8 (20)	7	−33.3	− .9
	2.0	10	61.1 (20)	6	+ 2.8	−1.4
	1.0	10	0 (20)	0	+11.4	+1.5
Phenobarbital§	.2	9	100.0 (20)	9	−10.3	− .8
	.1	9	37.9 (20)	0		− .8
Ethyl alcohol	20.0	9	92.0 (20)	7	−20.0	− .4
	10.0	9	28.0 (20)	3		+ .7
Atropine	.2	10	67.6 (20)	4	−17.7	+1.3
	.1	10	73.5 (20)	5		−1.7
SKF 501	1.0	10	88.6 (20)	7	−17.1	− .3
	.4	10	37.1 (20)	1		+ .3
	.1	10	20.0 (20)	1		+ .4

* Drug-diet conc. or, with alcohol, conc. in drinking water.

† No. in parentheses are No. of days used to determine % decrease in estrus, excluding first 3 days of diet administration.

‡ .4% conc. was tried but discontinued due to toxicity.

§ .3% *Idem*

all caused marked decreases in number of estrus cycles (68 to 100%) in doses causing no severe loss in body weight. Promazine HCl was less effective inasmuch as the decrease in estrus was associated with greater weight loss and fewer mice developed persistent diestrus.

A fairly prompt return of cycles occurred after cessation of therapy in most mice. This is indicated by figures in column 6, Table I, which compares the number of estrus cycles during a control period of 20 days immediately following drug administration with the control period preceding therapy.

Pregnant mare serum (Gonadogen), given subcutaneously to mice while still on diet, induced estrus in most mice on all diets (Table II). Doses of one or 2 international units (I.U.) were used. In mice on control diet, who were in persistent diestrus for no known reason, one I.U. induced estrus in 6 of 10 mice and 2 I.U. did so in 5 of 6 mice.

TABLE II. Effect of Pregnant Mare Serum in Diestrous Mice.

Drug	Conc. in %	I.U. of PMS*	Estrous/diestrous mice†
Reserpine	.001	1	4/6
	.001	2	8/9
Chlorpromazine	.3	1	8/8
Promazine	.2	2	2/3
Promethazine	.3	2	6/7
Phenaglycodol	1.0	2	8/8
Meprobamate	3.0	2	9/9
Phenobarbital	.2	2	9/9
Ethyl alcohol	20.0	1	6/8
Atropine	.2	2	4/7
SKF 501	1.0	2	7/8

* International units of pregnant mare serum.

† Ratio gives No. of mice developing estrus out of total No. in persistent diestrus.

That restriction in food intake sufficient to

TABLE III. Effect of Diet Restriction for 23 Days on Estrous Cycles and Body Weight.

Food given, g/day	Body wt lost, g	% decrease in No. of estrous cycles	No. mice
ad lib.	.3	0	6
4.0	1.8	21.6	10
3.5	3.5	40.0	9
3.0	4.5	56.4	10
2.5	6.9	67.6	10
2.0	9.9	91.7	10

cause severe weight loss will disturb the sexual cycle is well-known. Table III shows the effects of varying intakes of control ground food on body weight and estrus cycles of ZBC mice in our laboratory. A graph (not included) of these results gives essentially a straight line and shows that a 10% decrease in number of estrus cycles occurred/gram of body weight lost. Thus, decrease in incidence of estrus in mice on drug therapy (Table I) cannot be accounted for by weight loss, except possibly in the case of promazine. Also, no correlation between development of persistent diestrus in individual mice and their changes in body weight could be found. Mice in persistent diestrus frequently lost no weight or less weight than those with continuing cycles.

Mice were not handled except to do daily vaginal smears so only subjective impressions regarding any tranquilizing effect were obtained. Ptosis was observed in mice on reserpine. A mild sedation seemed to be present in mice on reserpine and phenobarbital. No definite effect was discernible with chlorpromazine, promethazine, phenaglycodol, meprobamate, alcohol, atropine or SKF 501. Promazine appeared to increase activity slightly.

Discussion. Interference with estrus can conceivably occur by many mechanisms. These experiments do not pin-point a site of action for the agents studied. However, induction of estrus by administration of pregnant mare serum suggests that a decrease in secretion of gonadotropin occurred. Such an effect could be explained by the theory of a hypothalamic regulation of gonadotropin secretion(5,6) in which both an adrenergic and a cholinergic link is involved(7).

Inhibition of estrus in mice by sedative and tranquilizing drugs could have its counterpart clinically for drug-diet administration simulates repeated oral administration of drugs. Disturbances in sexual cycles in the human do not appear to be a frequent finding but closer observation and questioning may reveal some changes.

Summary. Five tranquilizing drugs (reserpine, chlorpromazine, promethazine, phenaglycodol and meprobamate), 2 sedative drugs (phenobarbital and ethyl alcohol), an adrenolytic agent (SKF 401) and an anticholinergic drug (atropine) have been shown to cause persistent diestrus in mice when administered in food or water.

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Effects of Antibiotics on Synthesis of Lysine Decarboxylase in *Escherichia coli*.* (24032)

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This study is an extension of an earlier report that antibiotic chlortetracycline impairs, *in vivo* and *in vitro*, the elaboration of amines by intestinal microorganisms(1). Subsequently, others have noted similar effects (2-5). Since these amines arise through the action of the amino acid decarboxylases, a group of adaptive enzymes(6), and because a number of antibiotics inhibit the synthesis of proteins such as adaptive enzymes(7-10), it is conceivable that the induction of amino acid decarboxylases in bacteria is sensitive to certain antibiotics. The experimental work to follow supports this thesis, particularly insofar as chlortetracycline, oxytetracycline and chloramphenicol are concerned.

Methods. Production of cells. The bacterium, *Escherichia coli* (Crookes), was grown in a synthetic medium containing glucose (2%), KH_2PO_4 (0.1%), K_2HPO_4 (0.1%), NaCl (0.1%), $(NH_4)_2SO_4$ (0.4%), Na citrate (0.05%), $MgSO_4 \cdot 7H_2O$ (0.07%), ferric ammonium sulfate (1.56 mg%) and lactic acid (4 ml 80%/100 ml medium). The medium was adjusted to pH 6.8, dispensed in 100-ml lots in 250- or 500-ml Erlenmeyer flasks and sterilized at 114° for 10 min. The medium was usually inoculated with 0.1 ml of a 12-hr culture grown in the same menstruum. Unless otherwise stated, the inoculated medium was incubated at 25° for 18 hrs. To obtain cells with lysine decarboxylase activity, the medium had to be supplemented with L-lysine · HCl (0.02M). *Determination of enzymic activity.* The cells were harvested by centrifugation, washed once with distilled water and resuspended in 0.05M phthalate buffer, pH 4.5. Activity of lysine decarboxylase was determined according to Warburg technic by measuring CO_2 evolution from amino acid substrate at 30°. The side arm of each cup contained 0.5 ml 0.05M L-lysine · HCl in 0.05M phthalate buffer, while 1 ml of suitable density of cells (0.4 to 1 mg N) and sufficient buffer to achieve total volume of 3.2 ml were placed in the main compartment. The vessels were gassed for 7 min with a mixture of 95% nitrogen and 5% CO_2. After equilibration, the substrate was tipped into the main compartment and the increase in pressure noted at 5 or 10 min. up to 2 hrs. On occasion the cells, after being washed, were treated with toluene to increase permeability so that the effects of the coenzyme, pyridoxal phosphate, could be measured(11). This necessitated use of phthalate buffer at pH 5.5 instead of 4.5 for maximal activity. Pyridoxal phosphate was added at a level of 10 μg/cup. *Induction of lysine decarboxylase in resting cells. First method.* Cells were recovered from basal medium (no lysine present), washed and suspended in 0.05M phthalate (pH 4.5 or 5.5). To the main compartment of each Warburg vessel was added cells (0.4 mg N), glucose[‡] (50 mg) and pyridoxal phosphate (10 μg); the sidearm received 0.5 ml 0.05M L-lysine · HCl. After gassing with nitrogen, lysine was tipped in from sidearm and appearance of lysine decarboxylase activity observed over a 3-hr period at 30°. *Second method.* Because of abundance of CO_2 which evolved from glucose when induction was measured by the first method, a method was adopted which eliminated glucose during manometric measurement of lysine

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[†] The work reported represents a portion of thesis submitted May, 1956, by senior author to Graduate School of University of Minnesota in partial fulfillment of requirements for degree of Doctor of Philosophy. Present address: Elgin State Hospital, Elgin, Ill.

[‡] Mandelstam(11) found glucose to be essential for induction of lysine decarboxylase in resting cells of *Bacterium cadaveris*.

TABLE I. Effect of Oxytetracycline and Chlortetracycline on Formation of Lysine Decarboxylase in *Escherichia coli* during Growth.

		Activity (μl CO_2/hr) in cells from medium with:		
Nature of cell suspension (.4 mg N)	pH in cup	Oxytetracycline	Chlortetracycline	No antibiotic (control)
Intact	4.5	190	225	297
Toluene-treated	5.5	266	404	536

decarboxylase. Unadapted cells were obtained as before and suspended in buffer at a density equivalent to approximately 0.5 mg cell N/ml. Test tubes (20 x 200 mm), fitted with sidearm which could be closed with a rubber stopper as used with serum bottles, were arranged in a series for gassing with nitrogen. The following materials were added to these tubes: 10% glucose (2 ml), 0.128M L-lysine · HCl (2 ml), distilled water or solution of antibiotic (1 ml), 0.05M phthalate buffer at pH 5.5 (21 ml) and cell suspension (4 ml). The tubes were placed in water bath at 30° and gassed with nitrogen for 5 min. Four 5-ml aliquots, including one at the very beginning, were removed and promptly refrigerated at measured intervals during 1 hr. As soon as possible, the cells were washed by centrifugation and resuspended in phthalate buffer, pH 5.5. One ml of each suspension, equivalent to 1 ml cell N, was used to determine, by the customary manometric procedure, the activity of lysine decarboxylase.

Results. The Q_{CO_2}(N) of lysine decarboxylase in *E. coli* recovered from the lysine-supplemented defined medium was generally of an order of magnitude of 600. Toluenized cells were 2 to 3 times more active than intact resting cells. Pyridoxal phosphate increased activity of toluenized cells by less than 10%. Lysine decarboxylase, in either intact or toluene-treated cells, was completely stable in buffered, substrate-free suspensions of intact or toluene-treated cells for several hours in the Warburg vessel at 30° under nitrogen. This agrees with reported relative stability of this enzyme in cell-free preparations(12) and eliminated concern over spontaneous denaturation of the enzyme during subsequent tests. The tetracycline group of antibiotics had but slight effect on activity of lysine decarboxylase under conditions employed to measure the action of this enzyme. In fact, slight stimulation of activity was noted with intact resting cells.

Effects of chlortetracycline and oxytetracycline on formation of lysine decarboxylase in growing cells. Antibiotic was added to the lysine-fortified synthetic medium at a level which would not interfere with growth or acid production. This maximal non-inhibitory concentration of tetracyclines was 0.1 μg/ml. In one typical experiment (Table I), chlortetracycline · HCl or oxytetracycline · HCl, at a concentration of 0.04 μg/ml of medium, suppressed significantly the formation of the enzyme without a measurable influence on final pH or turbidity of the culture.

Effects of antibiotics on enzyme induction in resting cells. To determine the action of higher concentrations of antibiotic on formation of lysine decarboxylase necessitated the use of resting cells. Preliminary to studying effects of antibiotics on induction, however, the conditions optimum for induction of non-proliferating cells had to be established. Using the "first method," the effects of aeration and temperature during growth of unadapted cells as well as the pH of suspension during induction were tested. The organism was grown in the synthetic medium devoid of lysine on a rotary shaker at 30°, or without agitation at either 25° or 30°. The cells were recovered by centrifugation after 20 hrs of incubation, washed and resuspended in 0.05M phthalate buffer at pH 4.5 or 5.5. On the basis of results in Table II, cultivation without agitation at 25° and induction at pH 5.5 gave the best response. Under the conditions employed, the lag period for induction of this enzyme was less than 10 min. Also, it was learned that exogenous supply of pyridoxal phosphate was not influential on inductive process in resting cells except for a very slight reduction in length of lag period. The "first method" did not lend itself well to determin-

TABLE II. Effects of Aeration and Temperature during Growth on Subsequent Inducibility of Lysine Decarboxylase in Resting Cells of *Escherichia coli.*

Conditions during growth Extent of aeration	Temp.	pH in cup	Net activity (μl CO_2/3 hr)*
Agitation	30	4.5	8
"	"	5.5	24
Stationary	"	4.5	15
"	"	5.5	181
"	25	4.5	7
"	"	5.5	266

* .4 mg cell N/cup.

ing the influence of antibiotics on induction of lysine decarboxylase because of rapid production of CO_2 from the glucose present, which, incidentally, was impeded by chlortetracycline and oxytetracycline. The other method of testing the action of antibiotics on induction of lysine decarboxylase in resting cells was relatively successful. Unadapted cells were obtained from stationary cultures incubated at 25° and induced according to description of the "second method" (*cf. Methods.*). It is evident from the results in Table III that chlortetracycline and oxytetracycline discouraged induction of lysine decarboxylase. Of interest is the rapidity with which the cells responded to lysine as reflected in activity of control cells at zero time, presumably a result of adaptation while these cells were refrigerated to await removal of the last aliquot. In retrospect, this difficulty might have been alleviated by employing an inhibitor, *e.g.*, azide (11), one which would not affect subsequent activity of the enzyme induced. No alteration in the population of the cells could be detected turbidimetrically during the induction and holding periods.

TABLE III. Time Study of Action of Oxytetracycline and Chlortetracycline on Induction of Lysine Decarboxylase in Resting Cells of *Escherichia coli.*

Treatment	Length of induction period (min.)* 0	20	40	60
None (control)	79†	164	174	200
Oxytetracycline‡	48	58	61	44
Chlortetracycline‡	44	35	44	41

* An additional period of time elapsed between induction and measurement of activity. Undoubtedly this contributed to the final activity.

† Q_{CO_2} (N)

‡ Used at a conc. of 1 μg/ml during induction.

In further experiments, both anaerobic and aerobic conditions were maintained during induction period in testing a number of other antibiotics by the last-mentioned method. The results of these experiments (Table IV) indicate that chlortetracycline, oxytetracycline and, to a lesser degree, chloramphenicol interfered with induction of lysine decarboxylase. The other antibiotics were either slightly inhibitory or stimulatory to formation of this enzyme. Marked stimulation was observed with bacitracin and neomycin. Bacitracin caused pronounced clumping of the cells.

TABLE IV. Effect of Various Antibiotics on Induction of Lysine Decarboxylase in Resting Cells of *Escherichia coli.*

Antibiotic	Conc. (per ml)	Oxygenation during induction Aerobic	An-aerobic
Chlortetracycline HCl	1 μg	21*	39
	10 "	20	23
Oxytetracycline HCl	1 "	17	10
	10 "	17	11
Chloramphenicol	10 units	76	123
	100 "	4	6
Streptomycin	1 "	285	245
	10 "	194	240
K penicillin G	10 "	249	250
	100 "	256	200
Neomycin	10 "	320	300
	100 "	166	146
Bacitracin	10 "	320	300
	100 "	276	415
Control, initial†		22	18
" 90 min. later		240	245

* Q_{CO_2} (N) (after a 90-min. exposure to antibiotic).

† See footnote (*) in Table III.

Discussion. The results of this study contribute to the mounting evidence that certain antibiotics interfere with the metabolism of proteins(10,12,13,14). Thus chlortetracycline and oxytetracycline were found to inhibit induction of lysine decarboxylase in cultures of *E. coli* at levels having no measurable effect on growth. These observations suggest that syntheses of these proteins (enzymes) are singularly sensitive to the presence of such antibiotics. Also, exposure of resting cells to the 2 tetracycline compounds and chloramphenicol retarded induction of lysine decarboxylase; neomycin and bacitracin, under identical circumstances, were stimulatory to this process, while streptomycin and penicillin were without effect. Amino acid decarboxylases so affected need not be of the adaptive type since the production by *E. coli* of the constituitive enzyme, glutamic acid decarboxylase, has been reported to be inhibited by chloramphenicol as readily as the adaptive enzymes, arginine and lysine decarboxylase(2).

Although chlortetracycline, oxytetracycline and chloramphenicol blocked induction of lysine decarboxylase, it does not necessarily follow that they do so through an identical mechanism. The 2 tetracycline derivatives probably have a common mode of action(10). While chlortetracycline and oxytetracycline impaired the synthesis of this enzyme at comparable concentrations, chloramphenicol was active only at appreciably higher levels. The chelating property of tetracycline antibiotics may be significant since the inhibition of a number of enzyme systems by high concentrations of tetracyclines can be reversed by some divalent metal ions(15). Unfortunately the role of chelation was not studied, although low concentrations of these antibiotics were not inhibitory — rather, they were slightly stimulatory—to the activity of preformed lysine decarboxylase. In view of the hypothesis that amino acid decarboxylation results upon formation of a Schiff's base between pyridoxal phosphate and the substrate upon stabilization by a metal ion(16), higher concentrations of the tetracycline compounds might have reversed the activity of this enzyme. Sensitivity of the synthesis of lysine decarboxylase to these antibiotics may reflect interference with exergonic metabolism. Thus, tetracyclines have been reported to inhibit or uncouple oxidative metabolism(15,17,18) and to disrupt the electron transport system of DPN(19). It was noted in one phase of this study that the fermentation of glucose by resting cells was inhibited by the tetracyclines concomitant with interruption of the induction of lysine decarboxylase.

Efforts to divulge the "mode of action" of antibiotics are countless(20,21,22). While this study has not resulted in uncovering a mode by which some of the antibiotics inhibit the formation of adaptive enzymes, it does suggest avenues of approach which must await further developments in the field of the synthesis of inducible enzymes(14,23,24,25).

Summary. At concentrations not inhibitory to growth, the antibiotics chlortetracycline and oxytetracycline inhibited induction of lysine decarboxylase in proliferating cells of *Escherichia coli.* Induction of this enzyme in resting cells was readily inhibited by chlortetracycline, oxytetracycline and chloramphenicol. Penicillin and streptomycin were indifferent while bacitracin and neomycin enhanced induction of lysine decarboxylase in resting cells of this bacterium.

Addendum. After submission of this manuscript for publication, Bachrach, *et al.* (PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 874) reported tetracycline antibiotics to inhibit the formation of isoamylamine and isobutylamine (products of the adaptive enzymes, leucine and valine decarboxylases, respectively) in cultures of *Proteus vulgaris.* They reported also that high concentrations of tetracyclines interfered with the decarboxylation of tyrosine and phenylalanine in dried or cell-free preparations of *Streptococcus faecalis.*

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Temperature Effects on Botulinum A Toxin.* (24033)

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The physical and chemical properties of botulinum toxin(1,3,7,12,13,15,16,18,19, 20) and its physiological action(2,4,5,6,17) have been studied extensively because of the interest in the agent's high toxicity. Burgen *et al.*(4) found that toxin heated in a water bath at 100° for 15 minutes lost its ability to produce paralysis, and Ambache(2) showed that boiling it for 2 minutes destroyed its toxicity. In this report, more detailed data on the effect of temperature on type A botulinum toxin are presented.

Materials and methods. Five toxin preparations, supplied by Fort Detrick, Frederick, Md., were used. Two were partially purified and 3 had been crystallized. The initial studies were performed with the partially purified samples, designated S-1 and S-2. Inactivations at various temperatures and pH values were done with a crystalline sample labelled WA-19. The effect of heating on ultracentrifuge sedimentation patterns was determined with the remaining 2 crystalline samples. Toxicity was assayed in the goldfish, *Carassius auratus,* kept in aquaria at room temperature before and after inoculation. The technic has been described(5). The end points were calculated by modification(10) of the method of Reed and Muench (14) on the basis of mortality ratios observed after 36 hours. Thermal inactivations were carried out in a thermostatic water bath with temperature fluctuations of less than 0.1°. Since preliminary experiments showed that the toxin inactivated rapidly, the diluent, 0.1 M phosphate buffer at pH 6.9 containing 0.2% gelatin, was heated a few degrees above the desired temperature before mixing with the toxin, so that the mixture of toxin and diluent came to the inactivation temperature immediately. This eliminated the necessity of waiting for the mixture to come to thermal equilibrium before the first sample could be taken. Samples were removed from the reaction mixture at subsequent time intervals and tested in 10 goldfish. In this way, the effect of heating on the 50% lethal dose was determined.

Results. Preliminary experiments with

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the crude samples, S-1 and S-2, were performed at 50°. The first experiment showed that the toxin inactivated rapidly at first, but, after about 10 minutes, the inactivation rate slowed down. When the difference between LD_{50} at zero time and LD_{50} at time of heating, t, was plotted against time of heating, a curve was obtained which indicated the presence of at least 2 toxic components in the sample. The inactivation curves for both partially purified preparations were similar when heated under the same conditions, but the data were somewhat more scattered for S-2. Later work showed that endpoints obtained with goldfish were temperature dependent(5). Therefore, part of the data scatter was due to fluctuations in room temperature. Preparations S-1 and S-2 contained impurities, and it was believed that perhaps these impurities were toxic and might be one of the components seen in the inactivation curve. A crystalline preparation, WA-19, was obtained and the experiments were repeated. The inactivation curve produced by this more highly purified material had the same shape as the curves for the less pure preparations. WA-19 was inactivated at other temperatures. These temperatures were from 40° to 60° in 5° intervals. The results for 40°, 50° and 60° are shown in Fig. 1, and it can be seen that the toxin does not inactivate after heating for ½ hour at 40°. Other curves in Fig. 1 represent studies at 50° and 60°, and indicate a minimum of 2 components. These 2 components have different rates of thermal inactivation and the rates of each component were temperature dependent. By extrapolating the limb of the inactivation curve produced by the more slowly inactivating toxin to the zero time ordinate, it was found that this component constituted about 5% of the toxicity of the preparation. About 95% of the preparation inactivated rapidly. The data at 45° were too scattered to analyze, and those at 55°, while scattered, were entirely consistent with the interpretation of a 2-component system.

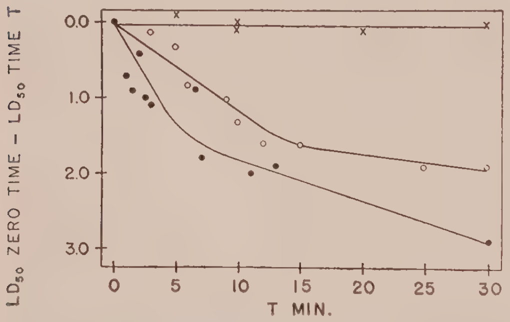

FIG. 1. Thermal inactivation curves for botulinum A toxin preparation WA-19. × = 40°; ○ = 50°; ● = 60°.

LD_{50} means logarithm to base 10 of the extent to which a toxin solution must be diluted to give 50% mortality when 0.1 ml is inoculated into each fish. Ordinates represent differences between logarithms of these dilutions for materials heated zero time and the times indicated on abscissa. It is possible to convert LD_{50} to the basis of No. of LD_{50}'s/ml or to the basis of No. of LD_{50}'s/mg nitrogen. However, such a conversion would not affect the difference between logarithms shown on ordinates. To convert the ordinates in Fig. 1 to logarithm of fractions of surviving toxicity, it is necessary only to change the sign of the difference between logarithms from plus to minus.

Since the presence of impurities would not account for the second toxic component, the nature of the 2 constituents was investigated. It has been shown that the toxic principle and the hemagglutinin of botulinum A toxin are separate entities(8,9,11). The hemagglutinin of WA-19 was removed by repeated absorption with chicken red blood cells. Parallel inactivation experiments on samples containing hemagglutinin and those not containing hemagglutinin were carried out. The end points, after heating at 50° for various times, were nearly identical, showing that hemagglutinin was not one of the components. The finding of Lamanna and Lowenthal(9) that hemagglutinin thermally inactivates more slowly than the toxic principle was confirmed in these experiments. The possibility that gelatin in the buffer might be affecting the shape of the curve was investigated, but the shape of the inactivation curve was the same in the presence and in the absence of gelatin.

The ultracentrifuge studies of Wagman and Bateman(19,20) and Wagman(18) on dissociation of botulinum toxin at high pH and ionic strength suggested the possibility that one of the effects of heat might be the dissociation of the toxin particle. Two small samples of toxin in acetate buffer at pH 3.8 were obtained and were run in the ultracentrifuge. Each produced a single sharp

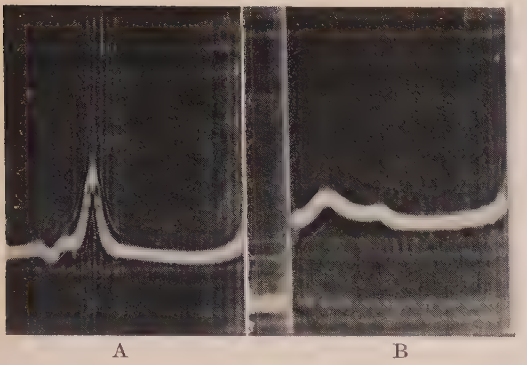

FIG. 2. Sedimentation diagrams for botulinum A toxin before and after heating. A. Toxin in 0.1 molar phosphate buffer at pH 6.9. B. Same sample after heating at 60° for 2 min.

peak. These samples were dialyzed against 0.1 M phosphate buffer pH 6.9 and the centrifuge analysis repeated. At pH 6.9, the sedimentation diagram showed 2 peaks, a small one which sedimented more slowly than a large peak which sedimented at the usual rate. Portions of these samples were heated for several minutes at the various temperatures used in inactivation experiments and again were run in the ultracentrifuge. After heating, the fast boundary became more diffuse indicating a possible increase in its inhomogeneity, and, in some cases, the slow boundary disappeared. Definite evidence for dissociation was obtained when the sample was heated at 60° for 2 minutes. When the sample was treated this way, the slow peak increased in area while the fast peak decreased in area and became more diffuse. The results are shown in Fig. 2.

Discussion. The results of these studies can be interpreted if it is assumed that the botulinum toxin particle can dissociate into 2 entities which have different thermal inactivation rates, and if it is further assumed that this dissociation can be produced by heating the particle. Wagman and Bateman (19,20) and Wagman(18) have presented evidence that the toxin molecule can dissociate into small toxic fragments when the ionic strength and pH of solvent buffer are increased. A similar experiment of ours confirmed their results. Changes produced in sedimentation diagrams of the toxin after it is heated, especially the diagram obtained after heating for 2 minutes at 60°, provide a basis for the second assumption.

The physical nature of these dissociation products might be postulated in at least 2 ways. Both of them might be fragments of the original particle or one might be the intact molecule and the other might be small dissociated parts of a molecule. The evidence does not permit the selection of one of these alternatives over the other.

Although the sedimentation diagram shows 2 components at pH 6.9, there is no evidence of 2 components when the toxin is inactivated at 40°. Either the components have the same inactivation rates at 40°, or the loss in toxicity is too small to be detected by the assay technic. The technic can detect about 2-fold differences in toxicity(5).

Summary. 1) Thermal inactivation of botulinum A toxin was studied. The LD_{50} was found for samples heated for various times at 40°, 50° and 60° by assaying samples in goldfish. Inactivation curves were plotted by plotting the difference between the LD_{50} at zero time, and the LD_{50} at time of treatment, t, against time of heating. At 40°, no inactivation occurred after heating one hour. At 50° and 60°, the inactivation curves were characteristic of a system which contained a minimum of 2 components which inactivated at different rates. Hemagglutinin was not one of these components and gelatin in the buffer did not affect the shape of the inactivation curve. 2) Sedimentation diagrams taken before and after heating at inactivation temperatures suggested that the toxin might be dissociating to form 2 particles having different properties. The data can be explained on the basis of this hypothesis.

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Variation in Virulence of Poliovirus. III. On Type III Virus by Plaque Method.[*] (24034)

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In a previous paper(1) experimental variation in mouse virulence of type III (Leon) poliovirus grown in tissue culture (TC) was reported and the influence of host cell and maintenance medium on viral virulence was demonstrated. The experiments have been extended, using the plaque technic. A mixed virus population and one purified by terminal dilution and by the plaque method were studied in regard to (a) distribution of virulent and avirulent progeny, and (b) reversibility of avirulent phase under influence of virulence enhancing media. The question as to whether the avirulent variant failed to multiply in the host or whether it multiplied without causing paralysis was also studied. The results are here reported.

Materials and methods have been described, (1) except for the following additions or modifications. The *plaque technic* of Dulbecco and Vogt(2,3) as modified by Hsiung and Melnick(4,5) was followed. Rhesus monkey kidney tissue cultures (MKTC) grown for approximately one week in Melnick's medium (0.5% lactalbumin hydrolysate in Hanks' salt solution with 2% calf serum), in 2-oz flat "prescription" bottles, were used. After removing the medium, each bottle was inoculated with 0.2 ml of virus dilution of known titer and incubated at 35°C for 45 minutes. Excess of inoculum was removed and the cells washed once with Hanks' salt solution. Approximately 4.5 ml of melted agar overlay (formula below) were added to each bottle. After agar had solidified, the bottles were turned over and incubated at 35°C for 3-4 days before plaque counts were made. *Agar overlay* was prepared as follows: Melted, sterilized 3% agar (Difco "Noble") in distilled water was mixed with equal portion of nutrient medium consisting of Earle's saline (10 x concentrated without either phenol red or $NaHCO_3$), 18.0 ml; calf serum, 4.5 ml; $NaHCO_3$, 5.4 ml of a 7.5% stock solution; neutral red (1:1000), 3 ml; antibiotics (penicillin 20,000 units/ml, streptomycin 10,000 mu/ml), 2 ml; and distilled water sufficient to make total volume of 90 ml. This mixture then contained 2.25 g of NaHCO/liter. *Virulence test in mice.* For assay of viral virulence, in addition to direct inoculation of TC fluid into mice as previously reported(1), the following procedure was followed: Well separated plaques were harvested with platinum loop, often from those bottles containing only

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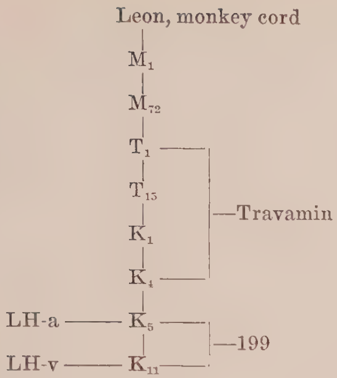

FIG. 1. History of LH-a and LH-v virus.

one plaque. Harvest from each plaque was suspended in 1 ml of Hanks' salt solution, and 0.2 ml of this suspension was inoculated into each of 2 or 3 MKTC tubes, with Hanks' salt solution and 2% calf serum as maintenance medium. This medium was considered as "neutral" because it neither enhanced nor reduced virulence for mice. Then 0.02 ml of pooled TC fluid of tubes from each plaque, (harvested usually on 4th day after inoculation) was inoculated intraspinally into each of 10 or more white mice, 4-5 weeks old. The $TCID_{50}$ of TC fluid usually varied from $10^{6.2}$ to $10^{6.8}$/0.2 ml. Virulence is defined here in terms of percentage of inoculated mice which developed paralysis; virulent, over 50%; avirulent, less than 10% and intermediate, 10-50%.

Results. The mouse-adapted Leon virus was serially passed in mice by intraspinal inoculation and from 72nd passage the virus was inoculated into monkey testicular TC (Fig. 1). All testicular TC and first 4 kidney TC passages were carried out in bovine plasma hydrolysate medium, while the following 7 kidney TC passages were carried out in 199 medium. As reported previously(1,6) the virus lost its mouse virulence after one or 2 passages in monkey testicular TC in plasma clot and bovine plasma hydrolysate medium but regained its virulence after further passages in MKTC in 199 medium. The avirulent passage K_5 was designated as LH-a while the virulent passage K_{11} was designated as LH-v (Fig. 1).

Plaque progeny of LH-v and LH-a virus. The K_{11} virus was plated and 10 plaques were picked. Each plaque was subcultured and the undiluted harvest of subculture of each plaque was titrated in tissue culture and in mice by intraspinal inoculation. Virus dosage/mouse varied from $10^{5.2}$ to $10^{5.8}$ $TCID_{50}$ but this variation seemed to bear little relation to outcome in mice. Among 10 plaques, 7 were virulent, 1 was avirulent and 2 were intermediate. Altogether, 105 mice were inoculated with material from 10 plaques and 63 (or 60%) of them became paralyzed. The avirulent virus of passage K_5 was also plated and subcultures of its 15 progeny plaques were tested in mice. It yielded 9 avirulent, 5 intermediate and 1 virulent plaque. Among 139 mice inoculated with virus from subcultures of these plaques, 10 (or 7%) of them became paralyzed.

Progeny of virulent and avirulent plaques. The question that now arose was whether virulent and avirulent plaques would breed true in a "neutral" medium. One of the virulent plaques from LH-v virus (K_{11}) was picked, subcultured, and replated. Ten progeny plaques were again assayed and they all were virulent. From other experiments an avirulent plaque was picked, and 3 serial plaque passages were made. From each passage 10 plaques were picked, subcultured and assayed in mice and 90% of progeny plaques of each passage were avirulent while the rest were intermediate forms.

Reversion of avirulent variants in Eagle's medium. From virulent LH-v virus (K_{11}), an avirulent culture (K_{12}) was isolated by terminal dilution passage (Fig. 2). This avirulent culture, after further purification twice by terminal dilution and once by plaque method, was again inoculated at its K_{17} passage level into mice as well as plated (Fig. 2). Direct inoculation of TC fluid into mice revealed that the virus was avirulent. By plaque analysis all of its 15 progeny plaques were also avirulent. To see whether this purified avirulent virus could regain its virulence in virulence enhancing media the virus was then serially passed in 2 sets of MKTC tubes, in Eagle's basal medium and 199 medium respectively. After 4 such passages the virus from either set was still avirulent. However after 7 passages (K_{24}) the virus from Eagle's medium became virulent while that from 199 medium was still avirulent. The viruses from both sets were plated and the one from Eagle's medium yielded 6 virulent out of 10 plaques while the one from 199 medium yielded only avirulent and intermediate plaques (Fig. 2). No effort was made to continue passages in 199 medium which was, as reported previously(1), also a virulence enhancing medium although not as effective as Eagle's medium.

The foregoing experiment, however, did not mean that all avirulent variants could easily regain their virulence in Eagle's medium. In the same experiment, among 15 avirulent plaques yielded by passage K_{17}, plaque a† and b† (Table I) were picked, subcultured and the virus from each of them was serially passed in 2 sets of MKTC, one in Eagle's medium and the other in bovine plasma hydrolysate. After 7 passages, virus from plaque a† regained its virulence in Eagle's medium but not in the other medium, while that from plaque b† did not regain virulence in either medium. (Table I).

The LH-a variant K_5, was also purified, first by terminal dilution 3 times and then

Key for Figures and Tables

T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. M = Mice inoculated intraspinally.

199 = 199 medium. Eagle = Eagle's basal medium. Travamin = Bovine plasma hydrolysate medium.

Te = Terminal dilution passage. Pq = Plaque passage. a, b, c, etc. = No. of individual plaque from which subculture was made for inoculation into mice.

V = Virulent; A = Avirulent; I = Intermediate.

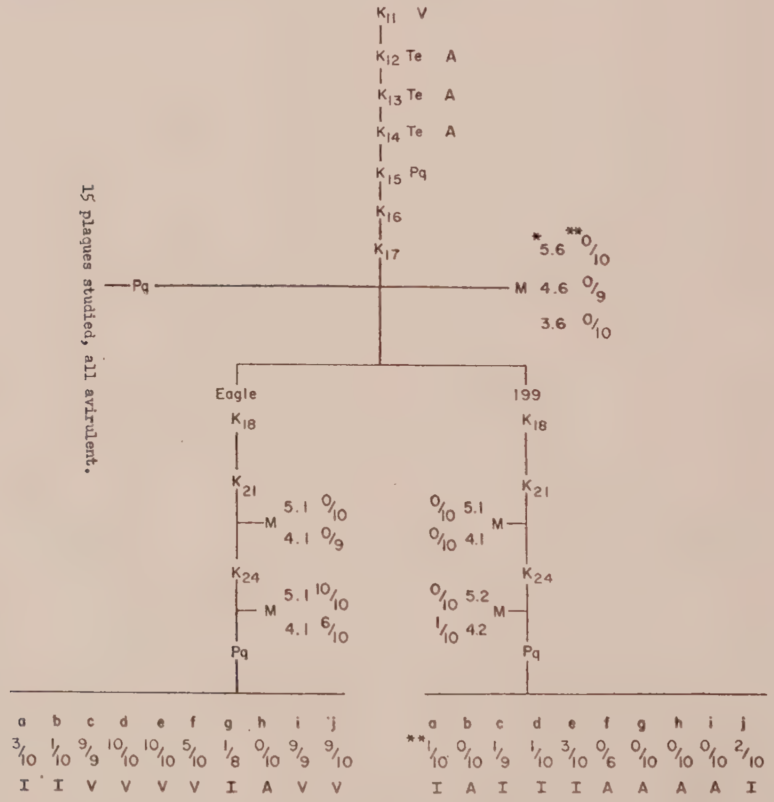

FIG. 2. Reversion of a purified avirulent variant in Eagle's medium.

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* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.
** No. of mice paralyzed over No. inoculated.

TABLE I. Reversion of Purified Avirulent Variants in Eagle's Medium.

Derived from	Passage or plaque No.	Virulence before purification	Purified by	No. of passages in Eagle's medium after purification	Result
LH-v, K_{11}	K_{12}	A	Te 3 Pq 1	7	V
	K_{17}, Pq a†	A	Te 3 Pq 2	7	V
	K_{17}, Pq b†	A	Te 3 Pq 2	7	A
LH-a, K_5	K_{11}, Pq c	A	Te 2 Pq 2	16	A
	K_{11}, Pq e	A	Te 2 Pq 2	27	A

once by the plaque method. Subculture from a single plaque was replated and 13 plaques were picked and subcultured. They were all avirulent. Virus from 2 plaques, c and e, was isolated and serially passed, each in two sets, in Eagle's and bovine plasma hydrolysate media, respectively. After 16 and 27 respective passages in Eagle's medium, the virus from both plaques still remained avirulent (Table I), as indicated by direct inoculation into mice at various passage levels and by plating for plaque analysis at end of experiment.

Recovery of virus from inoculated mice. At this point the question was asked: was the failure of the LH-a variant to paralyze mice due to its inability to multiply in the host? During study, spinal cords of some mice inoculated intraspinally with LH-a or LH-v virus were harvested, made into a 10% suspension in saline, centrifuged and supernates were then titrated in MKTC. The results are summarized in Table II. It is rather difficult to measure the extent of increase of virus in spinal cord. In the first place there was no way of knowing how much of the inoculum was absorbed by the spinal cord. Secondly, only the supernate of the cord suspension was used for titration while sediment which contained most of the virus according to our experience, was left out. Nevertheless an approximate comparison could be made. For the LH-v virus the average dosage of inoculum for each mouse was $10^{4.5}$ $TCID_{50}$ and the average $TCID_{50}$ per 0.02 g of the harvested cord tissue was $10^{4.4}$. For the LH-a virus the corresponding figures were $10^{5.5}$ and $10^{2.8}$ respectively.

TABLE II. Recovery of Virus from Spinal Cord of Paralyzed and Non-paralyzed Mice.

Virus inoculated	Mouse No.	$TCID_{50}$ (log 10) inoculated	Days after inoculation	Paralyzed or not	Log $TCID_{50}$ per 0.02 g of spinal cord tissue
LH-v	A	5.0	4	Yes	1.7
	B	5.0	4	"	4.8
	C	5.0	5	"	3.8
	D	5.0	5	"	4.5
	E	5.0	5	"	2.5
	F_1	5.2	5	"	4.3
	F_2	5.0	4	"	5.5
	F_3	5.0	8	"	4.7
	Group 1*	4.3	5	"	4.6
	" 2*	4.3	5	"	4.6
	" 3*	4.3	5	"	4.7
LH-a	A	5.8	4	No	2.7
	B	5.5	4	"	3.0
	C	5.5	5	"	2.5
	D	5.5	5	"	2.7
	F	5.5	6	"	2.2

* 5 mice.

Discussion. These experiments demonstrated that after purification some avirulent virus lines reverted to the virulent phase while others did not. The results suggest that purification by 3 or 4 plaque passages probably did not always yield a homogeneous population, which might explain why in some cases reversion occurred very easily. Or there might be a high back-mutation rate to virulence. It is noteworthy that 2 out of 3 avirulent plaques derived from LH-v reverted to the virulent state while 2 other avirulent plaques derived from LH-a did not revert.

Whether medium 199 in which LH-v had been serially passed was concerned in its genetic instability cannot be answered at present.

Titration in TC of the spinal cords of inoculated mice indicated that LH-a probably multiplied in the host to a lesser degree than LH-v, although both variants multiplied equally well in MKTC. In this respect they behaved like the virulent and attenuated strains of poliovirus in monkeys.

Summary. Both mouse-avirulent LH-a and mouse-virulent LH-v variants of Leon (Type III) poliovirus represented mixed populations, but LH-a yielded more avirulent plaques while LH-v yielded more virulent plaques. On replating, virulent and avirulent plaques usually yielded corresponding virulent and avirulent progeny, although intermediate forms sometimes appeared. After purification, virus from 3 avirulent plaques derived from LH-v was serially passed in Eagle's medium and in 2 of them reversion to virulence occurred; however, in another set of experiments, virus from 2 avirulent plaques derived from LH-a was passed in the same way and reversion did not occur. Titration in TC of suspension of the spinal cords of some inoculated mice indicated that LH-v multiplied more readily in the host than LH-a.

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Experimental Aminonucleoside Nephrosis.(I): Action of Cortisone on Aldosterone and Corticosterone Production. (24035)

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Singer(1) has shown that the nephrotic-like syndrome, induced in rats by an aminonucleoside related to puromycin(2,3) is accompanied by an increased rate of production of aldosterone as measured in the adrenal vein effluent. The purpose of the present work was to confirm this finding and to study the rate of secretion of aldosterone and corticosterone by the adrenals of normal and nephrotic immature rats treated with cortisone acetate.

Methods. Male hooded rats with starting body weight of 75 to 85 g were used. Nephrosis was induced by single daily subcutaneous injections of 6-dimethylaminopurine, 3-amino-d-ribose (1.75 mg in 0.2 ml of water/rat/day for 8 days). Cortisone acetate was given in single daily intramuscular injections for 5 consecutive days, beginning, in nephrotic animals, 24 hours after the last dose of the nephrotoxic drug. Unless otherwise specified, all animals were bled 16-18 hours after being last injected with either the nephrotoxic drug, or cortisone. Adrenal vein blood was collected according to operative procedure of Bush(4) under nembutal anesthesia (7.5 mg/100 g of rat). Since the rate of aldosterone and corticosterone secretion tends to fall markedly with time after one hour of bleeding (personal communication), the period of blood collection was limited to one hour. Blood from 2 to 5 rats was pooled and extracted as described by Bush(4). Residues were submitted to a single paper chromatographic separation in the benzene-aqueous methanol system(5), at room temperature (25 ± 2°C) 5 hours, after overnight equilibration. Corticosterone was eluted with methanol in a modified Haines' device(6), and quantitatively estimated, in duplicate samples, at 2400 Å according to the method of Saffran and Schalley(7). Spectrophotometric meas-

TABLE I. Aldosterone and Corticosterone in Adrenal Vein Blood of Normal Untreated Rats.

			μg/kg/adrenal/hr	
No. rats in pool	Mean wt, g	Mean vol blood collected, ml/adr/kg/hr	Aldosterone (as DOCA equiv.)	Corticosterone
3	90	24	33	94
3	93	24	43	105
3	84	24	20	92
4	93	23	38	91
5	90	22	23	80
4	101	24	25	89
Mean ± S.E.			30 ± 3.8	92 ± 8.5

urements of this steroid were performed in methanol solution against a paper blank. A standard curve using cortisone was established with each set of determinations. Corticosterone was furthermore characterized by soda fluorescence, reduction of blue tetrazolium, mixed chromatography and sulfuric acid chromogen. In the chromatographic system used, aldosterone is found in the section of the chromatogram extending from bottom of hydrocortisone to that of cortisone, as defined by mobility of these hormones used as pilots. In none of our experiments was aldosterone or any other Δ^4-3-ketosteroid present in this section in amounts sufficient to be detected under ultra-violet light (mineral light $\lambda = 2540$). This portion of each chromatogram was cut into small pieces which were soaked and shaken twice during 30 minutes with 20 ml of methanol. This eluate was brought to dryness and eventually bioassayed by the Simpson-Tait method(8). Its biological activity is hereafter referred to as aldosterone. In all instances the aldosterone content of normal and nephrotic rat adrenal vein blood was bioassayed and, at the same time that of the respective groups of animals treated with cortisone. In each assay the sodium retaining response of test animals was assessed by using 3 standard doses of desoxycorticosterone acetate. The level of aldosterone in rat adrenal vein blood is therefore expressed as desoxycorticosterone acetate equivalent (DOCA equiv.). Under the conditions of our experiment, preliminary results indicated that in this assay crystalline d-l aldosterone 21-monoacetate is more than 100 times as potent as desoxycorticosterone acetate. Following injections of the nephrotoxic drug, the development of experimental nephrotic syndrome was established on the basis of decreased serum albumin, changes in electrophoretic pattern of plasma proteins, hypercholesterolemia and ascites. In nephrotic animals, changes in electrophoretic pattern of plasma proteins were grossly characterized by marked decrease of albumin and elevation of α-1 globulin fractions. The magnitude of hypoalbuminemia and hypercholesterolemia was, in general, comparable to that reported by previous investigators(3).

Results. Secretion of aldosterone and corticosterone in adrenal vein blood of normal untreated rats is equal to 30 ± 3.8 μg DOCA equiv./kg/adrenal/hr and 92 ± 8.5 μg/kg/adrenal/hr respectively (Table I).

In the nephrotic animals, one series of rats was studied 16 to 18 hours and the other series 6 days after the last of 8 injections of aminonucleoside. The results are presented in Table II. Sixteen to 18 hours after last injection of the drug the nephrotic animals show a definite increase of aldosterone secretion as compared to that in normal animals (157 ± 9.3 μg DOCA equiv/kg/adr/hr: P ± .001), and a less striking but also significant increase of corticosterone secretion (143 ± 18.2 μg/kg/adr/hr: $P < .01$). The fact that these changes in adrenal secretion persist despite discontinuance of injections is proven by the finding of a comparable aldosterone (181 ± 29.3 μg DOCA equiv/kg/adr/hr) and corticosterone (172 ± 15.5 μg/kg/adr/hr) increase 6 days after withdrawal of the drug. In certain groups this is accompanied by a further increase of fluid retention as judged by greater accumulation of ascites (Table III).

When normal rats received cortisone at dose level of 100 to 500 μg intramuscularly/day for 5 days, the expected decrease of corticosterone secretion was noted (Table IV). Examination of mean values of corticosterone produced by animals receiving 350 and 500 μg of cortisone/day shows that injection of this hormone reduced the secretion of corticosterone to about ⅓ of values found in controls (Table I). Under cortisone administration the secretion of aldosterone was higher

TABLE II. Aldosterone and Corticosterone in Adrenal Vein Blood of Nephrotic Rats ("Early" Nephrotic).*

No. of rats in pool	Mean wt, g	Mean ascitic fluid, ml/kg body wt	Mean vol blood collected, ml/adr/kg/hr	μg/kg/adrenal/hr	
				Aldosterone (as DOCA equiv.)	Corticosterone
4	100	38	21	75	140
3	96	43	20	120	148
3	111	41	23	261	207
4	99	42	25	227	83
3	112	34	16	104	107
3	93	35	30	156	174
Mean ± S.E.		39 ± 1.5		157 ± 9.3	143 ± 18.2

* Nephrotic Syndrome induced by 6-aminonucleoside of puromycin: 1.75 mg/100 g body wt inj. subcut. daily 8 days. Animals were bled on ninth day: 16 to 18 hr after last inj. of drug.

Compared to that of normal rats the secretion of aldosterone by the nephrotic animals is very significantly increased: $P = .001$.

than the controls in all groups. Since the magnitude of the increase varies markedly with dose of cortisone and within different groups receiving the same dose, it is only of moderate statistical significance ($P = .02$). It may however be of interest to emphasize that, in spite of this increased aldosterone secretion which in 4 instances reached levels found in nephrotic animals, no signs of fluid retention were detected in these rats.

The action of cortisone on adrenal secretion of nephrotic rats is presented in Table V. At dose level of 350 and 500 μg/day, cortisone caused in 4 groups out of 7 a decrease of aldosterone secretion to normal values, whereas in the 3 remaining groups the hormone was depressed below normal in 2 groups and remained slightly above normal in one. Considered together, the values of aldosterone in the nephrotic series treated with 350 and 500 μg of cortisone are not significantly different from those of normal untreated animals ($P < .1$). Cortisone treatment of nephrotic animals, especially at the higher doses, resulted in a decrease of corticosterone secretion comparable to that observed in normal animals treated with identical amounts of cortisone (Table IV).

In the nephrotic series, administration of cortisone was accompanied by a marked decrease, or by disappearance of ascites. The values for ascitic fluid volume of nephrotic rats treated with 350 and 500 μg of cortisone/day are highly significantly different from those of untreated series measured either 16-18 hours or 6 days after last injection of the nephrotoxic drug ($P < .001$). Decrease in aldosterone secretion and in volume of ascites was not accompanied by changes in electrophoretic pattern of plasma proteins.

Discussion. The present results, although obtained with a different strain of animals,

TABLE III. Aldosterone and Corticosterone in Adrenal Vein Blood of Nephrotic Rats ("Late" Nephrotic).*

No. of rats in pool	Mean wt, g	Mean ascitic fluid, ml/kg body wt	Mean vol blood collected, ml/adr/kg/hr	μg/kg/adrenal/hr	
				Aldosterone (as DOCA equiv.)	Corticosterone
3	130	43	25	161	125
3	97	52	20	197	138
4	102	95	18	319	182
3	113	27	23	137	180
3	133	61	17	150	233
3	112	30	20	125	176
Mean ± S.E.		51 ± 10.2		181 ± 29.3	172 ± 15.5

* Animals were bled 6 days after last inj. of puromycin.

TABLE IV. Aldosterone and Corticosterone in Adrenal Vein Blood of Normal Rats Treated with Cortisone Acetate.*

Cortisone, µg/100 g body wt/day	No. rats in pool	Mean wt, g	Mean vol blood collected, ml/adr/kg/hr	µg/kg/adrenal/hr		
				Aldosterone (as DOCA equiv.)	Corticosterone	
100	4	94	23	60	70	
200	3	83	30	67	79	
350	3	84	27	130	37	32
"	4	92	18	—	28	
"	4	100	25	280	31	
500	2	95	20	126	27	17
"	4	97	19	74	16	
"	3	95	25	45	9	
"	4	87	23	75	13	
"	3	102	21	129	20	

* Cortisone acetate, Merck (lot #K.E. 207). Single daily intramusc. inj. for 5 days as aqueous microcrystalline suspension.

Animals were bled 16 to 18 hr after last inj. of cortisone. Compared to those of normal untreated controls the values of aldosterone of all cortisone treated groups are significantly elevated: P = .02.

fully confirm the finding of Singer(1) who showed that rats rendered nephrotic by 6-dimethylaminopurine, 3-amino-d-ribose present an increased rate of secretion of aldosterone. According to our results a less marked but appreciable increase of corticosterone secretion seems to be a rather constant feature of this experimental syndrome. However, the values of mean ratio aldosterone-corticosterone of 2 untreated nephrotic series (1.09 and 1.04) when compared to that of the normal animal (.34) emphasize that development of nephrosis is accompanied by a much higher rate of production of aldosterone than of corticosterone. One may point out that the observed changes in rate of secretion of aldosterone and corticosterone cannot be explained on the basis of a difference of blood flow through the adrenals, since in practically all our groups the mean volume of adrenal vein blood collected is quite comparable when expressed/adrenal/kg of body weight/hr.

The increased aldosterone secretion by normal rats treated with cortisone is an unexpected finding. Cortisone possesses some sodium retaining activity in the Simpson-Tait

TABLE V. Aldosterone and Corticosterone in Adrenal Vein Blood of Nephrotic Rats Treated with Cortisone Acetate.*

Cortisone, µg/100 g body wt/day	No. rats in pool	Mean wt, g	Mean ascitic fluid, ml/kg body wt	Mean vol blood collected, ml/adr/kg/hr	µg/kg/adrenal/hr		
					Aldosterone (as DOCA equiv.)	Corticosterone	
200	3	103	25	19	171	125	
350	3	115	24	17	27	68	69
"	3	110	traces	27	39	46	
"	3	98	"	24	7	93	
500	3	110	25	29	4	20	28
"	4	109	15	23	24	40	
"	3	100	16	20	53	32	
"	3	105	traces	21	22	21	

* Cortisone acetate, Merck (lot #K.E. 207) single intramusc. inj. daily for 5 days beginning 24 hr after last inj. of puromycin. Animals were bled 16 to 18 hr after last inj. of cortisone acetate.

Aldosterone values in nephrotic rats treated with 350 and 500 µg of cortisone are not significantly different from those of normal untreated controls.

Mean and stand. error of ascitic fluid vol of groups treated with 350 and 500 µg of cortisone: 12.3 ± 3.0.

bioassay(8). One could therefore expect that a certain amount of injected hormone is present in the general circulation and, assuming that it remains unchanged by passing through the adrenal, is therefore present in the collected adrenal vein blood effluent. If this was the case the chromatographic zone eluted for biological assay of aldosterone would contain cortisone (see methods). However, if one were to attribute to cortisone the sodium retaining activity observed, it would be necessary to assume the presence of this hormone on the chromatograms, in amounts readily detectable under ultra-violet light. It has already been pointed out that on none of the chromatograms did the aldosterone zone contain detectable amounts of Δ^4-3-ketosteroid material. It is therefore concluded that injection of cortisone in normal rats has led to an appreciable increase of aldosterone secretion. To a limited extent these findings are comparable to those of Farrell *et al.*(9) who showed that after long-term cortisone treatment the aldosterone secretion of a group of 4 normal dogs was increased moderately in 2 cases and appreciably in a third. However the overall aldosterone secretion in treated dogs, when compared to that of normal controls, proved to be statistically insignifiacnt. It is possible to assume that injection of cortisone in normal rats might in some way alter the electrolyte balance of animals, possibly by producing sodium diuresis, which, secondarily would be responsible for an increased output of aldosterone. At the present time this remains purely hypothetical.

Treatment of nephrotic animals with cortisone resulted in a decrease of rate of production of aldosterone and marked diminution of ascites. Since in certain groups the volume of ascitic fluid in untreated nephrotic rats increases sharply in the 6 days of observation following the last injection of the nephrotoxic drug (Tables III and IV), the action of cortisone in the nephrotic series may be interpreted both as decreasing ascites and preventing its further accumulation. These results are at variance with those of Fiegelson *et al.* (3) who were unable to demonstrate increased rate of production of aldosterone by the adrenals of aminonucleoside nephrotic rats, and to influence the degree of ascites by cortisone treatment. These discrepancies might in part be explained by the fact that these authors assessed the production of aldosterone of their animals by incubation of the adrenal glands, and that the dose of cortisone used by them was some 5 to 7 times higher than the effective doses used in the present study.

The present data do not provide information on the primary mode of action of cortisone in nephrotic rats in the sense that they do not disclose whether the decrease of aldosterone production is due to a direct action of cortisone at the adrenal level, or to its systemic action on the disease process, with secondary readjustment of mineralo-corticoid function. It is hoped that the results of further investigations will help to clarify this problem.

Conclusion. An increased rate of aldosterone production after induction of aminonucleoside nephrosis in rats has been confirmed. After short term treatment with cortisone acetate, at the dose level of 350 and 500 μg/day, corticosterone production of both normal and nephrotic animals was decreased to one-third of their respective controls. Cortisone reverted the increased aldosterone secretion of nephrotic rats to normal, this was accompanied by marked loss or even total disappearance of ascites.

The authors wish to express their deep gratitude to Dr. B. Singer who laid the foundation of this work in Montreal Children's Hospital, to Dr. S. Hardy from Lederle for a generous gift of 6-dimethylaminopurine, 3-amino-d-ribose; to Dr. F. Gross and Dr. C. W. Murphy, CIBA Co. for aldosterone; to Dr. J. H. Laurie, Merck Co. for cortisone acetate; to Dr. Frances McCall and Dr. Eleanor Harpur for friendly interest and help during this study. We are specially indebted to Miss A. Henry for the electrophoretic studies. This work has been supported by grants from Nat. Research Council of Canada and the Research Fund of Montreal Children's Hospital.

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Detection of Minute Gastrointestinal Bleeding Utilizing Radioactive Iron, Fe^{59}.* (24036)

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A simple method for detecting minute gastrointestinal bleeding would provide a screening test for detection of ulcerated lesions of the alimentary tract not recognizable by conventional radiographic or other currently available diagnostic means. Such a test could be utilized for detection of the presence of silent cancers of mucous membranes of the gastrointestinal canal, or possibly of other tracts with external orifices. The use of radioactive ferrous citrate, Fe^{59}, for this purpose was investigated. Radioactive iron given orally may make its appearance in red blood corpuscles as early as 4 hours following ingestion(1). Radioactive iron given intravenously is also rapidly incorporated into the red blood cell(2). Bleeding from the mucous membrane of the alimentary tract should be recognizable by the appearance of increased radioactivity in the stool provided normal excretion patterns of the isotope by this route are determined.

The experiments reported herein indicate that occult bleeding in the gastro-intestinal tract can be detected by the use of radioactive ferrous citrate incorporated into the red blood cell. After preliminary study, the method appears to be more sensitive than the guaiac test for this purpose.

Method. Twenty healthy mongrel dogs weighing 10-15 kg, and divided into 3 groups, were utilized in this study. Group I consisted of 8 control animals, each of which received 25 microcuries of radioactive ferrous citrate (Fe^{59}) intravenously. Group II consisted of 6 test animals. Each of these received 25 microcuries of radioactive ferrous citrate intravenously and after 14 days also received 30 mg of histamine in beeswax intramuscularly daily. Ulcerations of the gastrointestinal tract occur regularly in the majority of animals so treated(3). Group III consisted of 6 normal dogs. Each of these received by gastric tube small amounts of Fe^{59} tagged blood obtained from one of the Group I animals. In all animals of Groups I and II following injection of the isotope and in Group III dogs following intragastric injection of the labeled blood, daily stool collections were made. An equal amount of water was added to each 24-hour stool sample and mixed thoroughly in Waring Blendor. Four ml of resultant mixture was then counted in a well-type scintillation counter, 1000 or more counts being recorded for each specimen. Values are expressed as number of counts/4 ml of stool-water mixture for 10 minutes, which actually represents the number of counts/2 ml aliquot of stool. Whole blood was removed by venipuncture from animals of Groups I and II at intervals, and one ml was counted in the scintillation counter. The guaiac test for occult blood was

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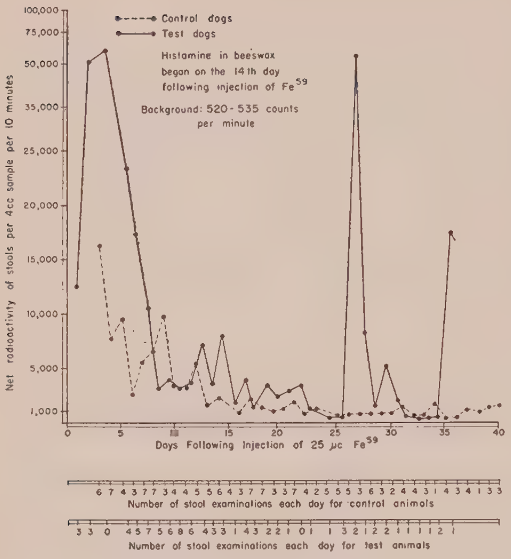

FIG. 1. Mean and extreme values of stool radioactivity in control and test animals.

also performed on all specimens. All animals were followed for 40 days or until death. During the study, all animals received regular kennel rations ("Kibbies") and no other food was given. Autopsy was carried out in all animals. Animals were excluded from Group I if intestinal parasites or other lesions were present, and no animals were included in Group II unless obvious mucosal ulceration of the gut was demonstrated later upon sacrifice. All animals fulfill the above criteria.

Results. Fig. 1 demonstrates the pattern of excretion of radioactive iron in the stools of Groups I and II dogs. Following injection of the isotope, prompt excretion through the gastrointestinal tract occurs; gradually, decreasing amounts are detectable in the stool of normal animals. After 21 days, excretion of radioactive iron by normal animals has reached consistently low levels. In none of 65 stool determinations done after this time were counts of more than 2500 above background recorded. Animals of Group II showed a similar pattern of excretion following injection of the isotope with gradually declining levels of stool radioactivity. However, following administration of histamine in beeswax, elevated excretion of the isotope in the stool was observed. Of a total of 45 stool determinations upon Group II dogs, performed 21 or more days following administration of the isotope, 17 showed counts greater than the 2500 level. Thirteen of these 17 were associated with a positive guaiac test and in 4 the guaiac test was negative. The radioactive iron method, in detection of an indeterminate amount of bleeding, was thus positive in 24% more of determinations than was the case with the guaiac test.

On the 24th day following injection of 25 microcuries of the isotope, counts of peripheral blood averaged 6675 counts/ml/minute for control animals and 5591 counts/ml/minute for animals receiving histamine in beeswax.

Data from Group III animals are shown in Table I. Amounts as small as 1 ml of Fe^{59} tagged blood given to animals by gastric tube were detectable by the ensuing high levels of radioactivity in the stool. Most of the blood was excreted within 24 hours of administration and stool counts fell to low levels promptly thereafter. Amounts of blood less than 10 ml were not detectable by the guaiac test when administered in this manner. From these data the radioactive iron method would appear to be at least 10 times more sensitive

TABLE I. Stool Radioactivity following Intragastric Administration of Fe^{59} Tagged Blood in 6 Animals.

Amt of blood given (ml)*	Net stool activity in counts/4 ml/10 min.	Days after blood given	Guaiac test
10	4,867	1	0
	10,606	2	+
	835	3	0
	5,765	4	0
5	5,212	1	0
	40,607	2	0
5	1,244	1	0
	151	2	0
	214	3	0
3	12,605	1	0
	1,489	2	0
	46	3	0
1	13,517	1	0
	910	2	0
	1,004	3	0
1	1,822	1	0
	72	2	0
	922	3	0

* Radioactivity of blood given: 6,540 counts/cc/min.

Background count: 520-535 counts/min.

than the guaiac test for detection of known minute amounts of blood in the stool.

Discussion. Not all of the injected radioactive iron is incorporated into red blood cells. Hahn and associates(4) have shown that 35 to 70% of injected isotope becomes incorporated in peripheral circulating red blood cells. However, if a sufficient period of time is allowed to elapse following injection of radioactive iron, the excretion pattern of the isotope in the stool may subsequently be utilized for detection of bleeding from the alimentary canal, since elevated levels of stool radioactivity after 21 days are indicative of abnormal sources of iron loss.

The method appears to be considerably more sensitive than the guaiac test, utilizing amounts of the isotope and the technic for determination of stool radioactivity described herein.

It is conceivable that the sensitivity of the method might even be enhanced somewhat by modifying the technic of examination by ashing and counting the entire 24-hour stool. If proved correct, this modification could permit administration of smaller amounts of the isotope. In the presence of a positive test, a comparison of esophageal, gastric and colonic washings could prove to be of real help in localizing the source of the bleeding from the tract. It is proposed presently to undertake clinical investigation of the efficacy of the method in the detection and localization of silent malignancies of the gastrointestinal canal.

Summary. 1. Excretion patterns of intravenously administered radioactive iron in the stool of normal dogs and in dogs with histamine induced bleeding of the gastrointestinal tract have been compared. 2. Levels of stool radioactivity have been measured following intragastric administration of known amounts of isotope-labelled blood. 3. Radioactivity in the stool can be utilized for detection of occult bleeding. 4. Comparative evaluation following administration of known small quantities of isotope-labelled blood (Fe^{59}) indicates definitely that this test is considerably more sensitive than the guaiac test.

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Pressor Substances in Kidneys of Renal Hypertensive Rats with and Without Adrenals. (24037)

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In the rat, sustained hypertension develops after clamping the artery of one kidney, leaving contralateral kidney untouched(1). It has also been demonstrated that the content of pressor substances (renin) in aqueous extracts prepared from kidneys is normal or enhanced in the clamped kidney, while it is reduced or absent in the contralateral untouched kidney which, however, shows typical morphological changes(2). Furthermore, it is well known that experimental renal hypertension of the Goldblatt type is dependent on presence of functioning adrenal cortex. In the adrenalectomized animal, hypertension does not develop or blood pressure falls to normal values if the adrenals are extirpated after establishment of hypertension(3). In former experiments we observed that in rats in which hypertension has been produced by overdosage of cortexone and salt, pressor active substances disappeared from the kidneys (4). The experiments reported here were

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* We are indebted to Mr. R. Doebelin for technical assistance.

performed in order to evaluate the significance of unequal distribution of renin in the clamped and unclamped kidney in development of renal hypertension and to study the influence of adrenalectomy on the renin content of the kidneys.

Methods. Male Wistar rats with average initial weight of 150-200 g were used. Food consisted of standardized pellets containing 0.57% of salt. Tap water or 0.5% saline was given as drinking fluid. A. *Production of renal hypertension.* Two methods were used: clamping of left renal artery or encapsulation of left kidney by cellulose acetate. Clamps were prepared from silver strip, bent over metal plate of 0.2-0.3 mm thickness, the space within the clamp being made proportional to size of animal. For the encapsulation, animals were anesthetized with ether, the left kidney exteriorized through a paravertebral incision. A thin leaf of cellulose acetate (0.1 mm thickness) bearing a hollow for kidney pedicle was placed around kidney and kept together by 2 rings of rubber. After hypertension had developed in the majority of animals in both groups, they were killed by ether. Kidneys were removed and the pressor material determined by the method described below. B. *Adrenalectomized animals.* Two groups of 7 animals each were used. In all animals the left renal artery was clamped. In one group the adrenals were removed a fortnight after clamping and after hypertension had developed. The other 7 animals were sham operated. After adrenalectomy, animals received 0.1 mg cortisol acetate subcutaneously/day. Five weeks after beginning of experiment, *i.e.,* 3 weeks after adrenalectomy or sham operation, all animals were killed and kidneys assayed for their pressor activity. Blood pressure was determined by tail plethysmograph method under light ether anesthesia. C. *Bioassay of pressor material.* Kidney extracts were prepared by grinding the minced kidney with sand and isotonic saline. Ground tissue was centrifuged 30 minutes at 6000 rpm. Such extracts contain the pressor active material in the supernatant fluid. The pressor activity was determined semiquantitatively in nephrectomized rats anesthetized with urethane (1.4 g/kg s.c.) from which kidneys were removed about 20 hours before. A standard dose of 0.1 ml of aqueous kidney extract was injected into jugular vein. Results were judged by maximum blood pressure and the integral of blood pressure curve over 30 minutes expressed in cm^2.

Results. A. Pressor activity in clamped or encapsulated and in contralateral intact kidney. In nephrectomized test animals, average increase in blood pressure produced by 32 extracts of an equal number of normal kidneys was 64.8 ± 2.1 mm Hg. Blood pressure did not return within several hours to the level before injection. In the same animals intravenous injection of 0.02 mg/kg of adrenaline gave a short hypertensive response averaging 50.5 ± 2.2 mm Hg.

Hypertensive activity of extracts from

TABLE I. Pressor Activity of Freshly Prepared Extracts from the Unclamped (Right) and Clamped (Left) Kidney of Rats with Hypertension and with Normal Blood Pressure on 152nd Day after Operation. Maximal pressor response in mm Hg and integral of blood pressure curve in cm^2.

No. of rats	Final body wt (g)	Final blood pressure (mm Hg)	Kidney wt (mg)		Pressor activity (mm Hg) of extracts from unclamped (right) and clamped (left) kidney		Planimeter (cm^2)	
			Right	Left	Right	Left	Right	Left
12	198.7 ± 9.4	205.0 ± 6.9	955.0 ± 80.0	487.0 ± 38.0	17.4‡ ± 3.1	71.8† ± 4.6	13.4 ± 2.8	39.9 ± 2.9
19	202.1 ± 11.2	123.4 ± 4.9	1027.0 ± 44.8	358.0 ± 21.1	29.1‡ ± 4.2	68.2* ± 3.4	20.2 ± 3.2	35.6 ± 2.2

* $p < 0.3$
† $p < 0.2$
‡ $p < 0.001$
compared with content of pressor material of normal kidneys leading to rise of 64.7 ± 2.1 mm Hg (37 rats).

TABLE II. Pressor Activity of Freshly Prepared Extracts from Unclamped (Right) and Encapsulated (Left) Kidney at 80th Day after Operation. Maximal pressor response in mm Hg and integral of blood pressure curve in cm². In 2 groups the contralateral kidney was removed.

No. of rats	Final body wt (g)	Final blood pressure (mm Hg)	Kidney wt (mg)		Pressor activity (mm Hg) of extracts from unclamped (right) and encapsulated (left) kidney		Planimeter (cm²)		Drinking fluid	
			Right	Left	Right	Left	Right	Left		
6	164.7 ± 6.6	137.5 ± 14.5	681.0 ± 58.0	431.0 ±122.0	10.5‡ ± 6.0	61.0* ± 7.0	7.9 ± 4.0	41.0 ± 9.0	Water	
5	168.0 ± 4.0	118.0 ± 8.0	731.0 ± 26.2	455.0 ± 84.6	20.0‡ ± 5.8	47.2† ± 5.0	14.1 ± 6.8	29.5 ± 3.2	.5% NaCl	
6	159.0 ± 3.7	126.0 ± 7.1		736.0 ± 37.8		36.0‡ ± 12.6		25.0 ± 8.8	Water	**
6	150.0 ± 17.6	153.3 ± 4.4		826.0 ± 26.8		44.0‡ ± 2.4		33.0 ± 1.8	.5% NaCl	

* $p < .7$
† $p < .01$ } Compared with content of pressor material of normal kidneys.
‡ $p < .001$

** Unilaterally nephrectomized.

clamped kidneys was either normal or slightly enhanced, it was significantly below normal in extracts prepared from unclamped kidneys (Tables II and III). This difference in the content of pressor material in clamped and in contralateral kidney was more pronounced in animals in which hypertension developed (Table I).

Encapsulation of kidney produced results like those of renal arterial clamping. Administration of 0.5% saline as drinking fluid instead of tap water, did not lead to more pronounced decrease of pressor substances in the intact kidney but to a slight diminution of pressor activity in the encapsulated kidney. After nephrectomy the remaining encapsulated kidney contained less pressor material than a normal kidney, regardless of whether tap water or saline was given as drinking fluid.

B. *Influence of adrenalectomy.* In confirmation of results of others, adrenalectomy in renal hypertensive rats was followed by a

TABLE III. Body Weight, Blood Pressure, Kidney Weight and Pressor Activity of Freshly Prepared Extracts from Unclamped (Right) and Clamped (Left) Kidney of Renal Hypertensive Rats with and without Adrenals, Assayed in Nephrectomized Rats. Maximal pressor response in mm Hg and integral of blood pressure curve in cm².

No. of rats	Body wt (g)			Avg blood pressure (mm Hg)			Kidney wt (mg)		Pressor activity (mm Hg) of extracts from unclamped (right) and clamped (left) kidney		Planimeter (cm²)	
	Initial	14th day	35th day	Initial	14th day	35th day	Right	Left	Right	Left	Right	Left
7	205.1 ± 3.8	218.4 ± 3.4	256.5 ± 4.8	112.1 ± 4.2	177.1 ±10.5	210.7 ±10.2	1040.0 ±54.9	840.0 ±31.3	14.0§ ± 6.6	71.6* ± 6.9	16.3 ±10.0	46.3 ±5.5
6‖	204.3 ± 5.5	219.8 ±11.3	175.2 ±12.8	108.3 ± 3.1	172.5 ± 7.8	105.8 ± 4.2	897.0 ±34.5	393.0 ±45.8	54.8† ± 7.1	77.7‡ ± 3.9	39.8 ± 5.2	42.0 ±2.3

* $p < .3$
† $p < .2$ } compared with content of pressor material of normal kidneys.
‡ $p < .02$
§ $p < .001$

‖ Rat No. 3897 on autopsy showed surviving remnants of adrenal tissue. The following values for this rat were not included in calculation of the mean: Final blood pressure: 165 mm Hg; kidney wt: left, 949 mg; right, 1327 mg; rise in blood pressure of extract of left kidney: 58 mm Hg; of right kidney: 6 mm Hg. Final body wt: 236 g.

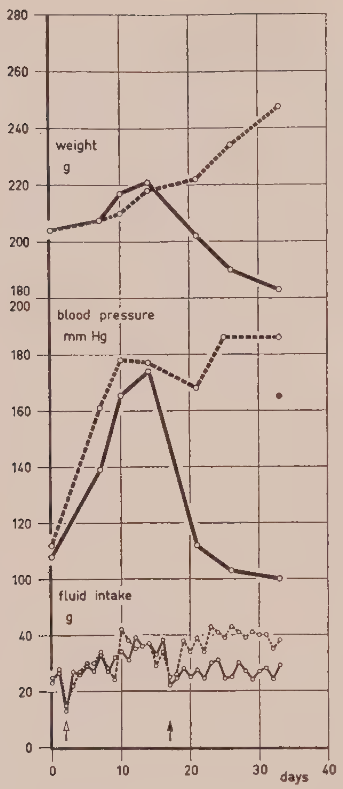

FIG. 1. Mean values of body wt (above), blood pressure (middle) and water intake (below) in rats with unilateral clamping of renal artery (dotted line) and in a second group adrenalectomized 17 days after clamping and treated with .1 mg of cortisol acetate daily. The point in blood pressure curve at 32nd day marks blood pressure of one animal of the adrenalectomized group with regenerated remnants of right adrenal. White arrow: clamping of left renal artery. Black arrow: bilateral adrenalectomy.

fall of blood pressure to normal values (Fig. 1). The content of pressor material in the clamped kidney was almost the same as in non-adrenalectomized animals, while in the intact kidney it was only slightly reduced and significantly higher than in kidneys from the corresponding sham-operated animals or from unilaterally clamped animals with intact adrenals. One animal of the adrenalectomized group (Table III) not only showed no fall in blood pressure but also complete depletion of pressor material from its right kidney. Autopsy revealed that adrenalectomy was incomplete in this animal.

The clamped left kidneys of the adrenalectomized animals were only about 50% of the weight of those with adrenals. The unclamped kidney, however, was only slightly smaller in the adrenalectomized animals than in animals from which adrenals were not removed. In spite of this difference in size, the pressor activity of kidney extracts prepared from these 2 groups are in the same range (Table III).

Discussion. In rats in which the left renal artery is clamped, in all animals, regardless of whether hypertension resulted or not, the content of pressor material in the clamped kidney was found to be either normal or slightly enhanced, while it was significantly reduced in the unclamped kidney. The same change in the distribution of pressor material was also observed if one kidney was encapsulated. The decrease of pressor material (renin ?) in the intact kidney is not strictly correlated with sustained high blood pressure. In hypertensive animals the removal of the adrenals not only produces a fall in blood pressure to normal values but is also followed by a restoration of the content of pressor substances in the right kidney. Whether higher doses of cortisol or other cortical steroids can prevent not only the fall in blood pressure but also the return to normal levels of pressor substances in the unclamped kidney has not been investigated. The fact that both blood pressure and the renin content of the unclamped kidney are simultaneously restored to normal by adrenalectomy makes it probable that the reduction of renal pressor activity is involved in pathogenesis of renal hypertension. It is, however, not a prerequisite, since sustained renal hypertension develops even more regularly in unilaterally nephrectomized animals bearing one clamped kidney. Hence it may be that depletion of renin-like substances and sustained renal hypertension are independent and unrelated phenomena.

Summary. 1. In rats, clamping of one renal artery or encapsulation of one kidney leads to a significant decrease in pressor material (renin ?) in the contralateral "untouched" kidney, while in the clamped kidney pressor activity is normal or only slightly enhanced. 2. These changes in content of renal pressor material are not necessarily accompanied by increase in blood pressure but were observed in every case of renal hypertension.

3. When hypertensive rats were adrenalectomized and maintained on small doses of cortisol, there followed a decrease in blood pressure and restoration of level of pressor material in the unclamped kidney. 4. It is suggested that diminution of pressor material in the unclamped kidney is not a prerequisite but a consequence of the pathological situation leading to renal hypertension.

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Use of Dyes with Rapid Bloodstream Clearance for Serial Determination of Cardiac Output. (24038)

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The T-1824 dye dilution method has been widely used for measurement of the cardiac output. Because of albumin binding the dye leaves the bloodstream very slowly. This causes serious technical and clinical difficulties when repeated injections of the dye are given for serial estimation of the cardiac output, for example, appearance of bluish coloration of skin and subcutaneous tissues, and development of excessively high concentrations of dye in the plasma above the range of accurate determination with a spectrophotometer. These facts suggested that a dye with rapid bloodstream clearance might be more suitable than T-1824 dye for frequent serial determination of cardiac output. The present study is concerned with investigation from this viewpoint of a group of 45 blue dyes.

Materials and methods. Healthy dogs weighing 10 to 25 kg were fasted 48 hours before each experiment. Anesthesia was produced by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight), after which an endotracheal tube with inflatable cuff was placed in the trachea. The dyes dissolved in distilled water and sterilized by heating were injected (0.25 mg/kg body weight) into one exposed femoral vein. Venous blood samples were removed from the opposite femoral vein before injection and at intervals after, *viz.* 1, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 minutes. Optical density readings of dye-plasma samples were made at point of maximum spectral absorption with Beckman Spectrophotometer, DU model. Spectral absorption curves and disappearance rates of 38 of the 45 blue dyes were investigated. The remaining 7 blue dyes were either partially soluble in water (Acetin Blue) or insoluble (Alcian Blue, Sudan Blue, Ultramarine Blue, Oil Blue, Galamine Blue, Alazarin Blue S) and were, therefore, unsuitable for further study. In the cardiac output experiments, the selected dyes were injected (0.15 mg/kg or 0.25 mg/kg body weight) into one femoral vein. Following injection, arterial blood samples were collected (from a polyethylene tube inserted through one femoral artery into the aorta) at 2-second intervals into cuvettes of motor-driven sample collector. Cardiac output values were calculated from formula of Stewart(1), of Hamilton and associates(2) as modified by Nicholson and Wood(3).

Results. Dyes cleared within one min. The following dyes were cleared from the bloodstream in less than one minute; Anthracene Blue, Toluidin Blue O, Victoria Blue R, Night Blue, Poirrer Blue, Prussian Blue, Luxon Fast Blue MBS, Niagara Blue 2B, Delphine Blue, Isamin Blue, Cyanin Blue G, Fast Acid Blue, Brilliant Cresyl Blue, Brilliant Alazarin Blue, and Azure Blue B.

Dyes cleared within 5 min. The following dyes were cleared between one and 5 minutes; Acid Alazarin Blue 2B, Nile Blue, Toluylene

TABLE I. Dyes Cleared from Bloodstream within 120 Min.

Dye		Max spectral absorption, mμ	Time of complete clearance from bloodstream, min.
Patent	Blue A	640	< 25
Brilliant	”	630	”
Methylene	”	670	< 20
Chicago	” B	580	< 15
Diamond	” 3B	520	< 10
Thymol	”	440	< 10
New Methylene	” N	580	< 15
Oxamine	” 4R	550	< 60
Trypan	”	600	<120
Azo	”	560	”
Patent	” V	640	”

Blue, Rhoduline Blue 6G, Celestine Blue, Alazarin Blue GS, Aniline Blue WS, Dianil Blue 2R, and Victoria Blue.

Dyes cleared within 120 min. Four dyes in this group (Table I) have maximum spectral absorption of 630 mμ or above, namely Patent Blue A, Patent Blue V, Brilliant Blue, and Methylene Blue. The plasma optical density readings produced by injection of Patent Blue A, Patent Blue V, and Brilliant Blue were much higher than those produced by equal amounts of Methylene Blue. Accordingly, we used Patent Blue A, Patent Blue V, and Brilliant Blue for determination of cardiac output.

Dyes cleared after 120 min. Because of their slow clearance from the bloodstream the following dyes were considered unsuitable for repeated injection at short intervals of time: Direct Sky Blue, T-1824, and Niagara Sky Blue.

Comparative values for cardiac output. Values for cardiac output obtained in dogs with selected dyes are comparable (Table II). Despite the fact that the paired determinations were not performed simultaneously, the estimated values are close.

Discussion. The following criteria were used in selecting dyes most suitable for repeated injection into the bloodstream for purposes of serial determination of cardiac output: (1) The dye should have a bloodstream clearance rate of approximately 20 to 30 minutes. However, dyes with longer clearance rates up to 120 minutes may be used. (2) The dye should reach and maintain a high concentration in the blood during the first minute after injection. (3) The maximum spectral absorption of the dye should be higher than that of hemoglobin, preferably, 630 mμ or above. In this way, hemolysis in a plasma dye sample will produce minimal interference with spectrophotometric determination of dye concentration. In addition, it is essential to establish that the selected dye is non-toxic and that solutions of the dye obey Beer's Law. Too rapid clearance of a dye from the bloodstream may not permit an adequate build-up of dye concentration to occur during the initial period when cardiac output is being determined. Of the 38 dyes studied, only 3 fulfilled the criteria listed above.

Summary and conclusions. Bloodstream clearance rates and spectral absorption curves of 38 blue dyes have been investigated. The results suggest that suitable dyes for frequent serial estimation of the cardiac output are Patent Blue A, Brilliant Blue, and Patent Blue V. These dyes leave the bloodstream rapidly after injection; they have an adequate build-up of concentration during the first minute; and their maximum spectral absorption occurs at wave lengths of 630 mμ or higher. Cardiac output values obtained in dogs with Patent Blue A, Brilliant Blue, Patent Blue V,

TABLE II. Comparison of Cardiac Output Values Obtained by Use of Patent Blue A, Patent Blue V, Brilliant Blue, and T-1824 Dyes.

Dog wt, kg	First cardiac output			Second cardiac output (30 min. later)		
	cc/min.		mg/kg	cc/min.		mg/kg
13.6	3090	Patent Blue A	.25	2840	T-1824	.25
13.2	2752	Brilliant Blue	.15	2993	T-1824	.15
11.8	3068	Patent Blue A	.15	3024	Patent Blue V	.15

and T-1824 dyes are presented and compared.

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Foreign-Body Type Reaction in Fat Cells.* (24039)

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During studies of the response of rats to diets containing very high amounts of saturated fats, a foreign-body type reaction was observed in fat cells of adipose tissue adjacent to sections of various organs prepared for histological examination. This report describes these altered fat cells and various dietary conditions under which they occur. Although a similar abnormality had been observed earlier with one of these fats by Cox and DeEds,† their data have not been published.

Methods and materials. Weanling male and female rats were obtained from Holtzman Rat Co. and fed one of the following diets. Each diet contained 20% vitamin-test casein, 4% B-vitaminized casein(1), 5% USP XIV salts, 4% corn oil, 17% glucose, and supplements of Vit. A, D, and E at levels of 40,000 I.U., 4,000 I.U., and 510 I.U./kg diet, respectively. The saturated fats incorporated at a level of 50% were fully-hydrogenated lard, palmitic acid, stearic acid, or a saturated partially acetylated monoglyceride(1). Ethyl stearate was fed at 45% with 5% cellulose added to provide body to the diet. A control group was given 50% lard. Rats were sacrificed at 8-week intervals and examined grossly. Portions of perigastric, perirenal, periadrenal, perigonadal, and subcutaneous fat were removed from each rat, fixed with Bouin's solution, sectioned, and stained with hematoxylin and eosin.

Results. Gross examination of the viscera and body cavities was not remarkable except for general fullness of the gastrointestinal tract with creamy colored food and an occasional mottled appearance of fat depots in animals fed the saturated fats.

Microscopic examination of sections showed an unusual foreign-body type reaction in fat from rats fed saturated fats (Fig. 1, top). The lesion consisted of a multinucleated cellular reaction usually located within the fat cell membrane with nuclei arranged evenly about periphery of the fat cell or in clumps. In many instances, the material which occupied the center of the fat cell, appeared to have been partially dissolved, leaving delicate slit-like spaces which were radially oriented and suggestive of fatty acid crystals (Fig. 1, bottom). The reactive process usually involved 2 or more adjacent fat cells and was separated from similar areas by uninvolved fat cells. No acute inflammatory reaction, evidence of recent hemorrhage, or birefringent material were associated with the fat reaction.

The phenomenon occurred within 8 weeks in rats fed diets containing palmitic acid, stearic acid, ethyl stearate, or saturated partially acetylated monoglycerides and within 16 weeks in rats fed the fully-hydrogenated lard diet, but was not observed in rats fed the control lard diet. The flecks of opaque white material observed grossly in the depot fat of some rats were associated with the severest occurrence of the foreign-body type reaction as seen microscopically.

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* Communication No. 247 from Research Labs., Distillation Products Industries.

† Observed in rats fed saturated acetylated monoglycerides for long periods of time by A. J. Cox, Dept. of Pathology, Stanford Univ., Calif., and F. DeEds, Western Regional Research Laboratory, Albany, Calif., private communication. *Added in proof*: Recently published by Cox and DeEds, *Am. J. Path.*, 1958, v34, 263.

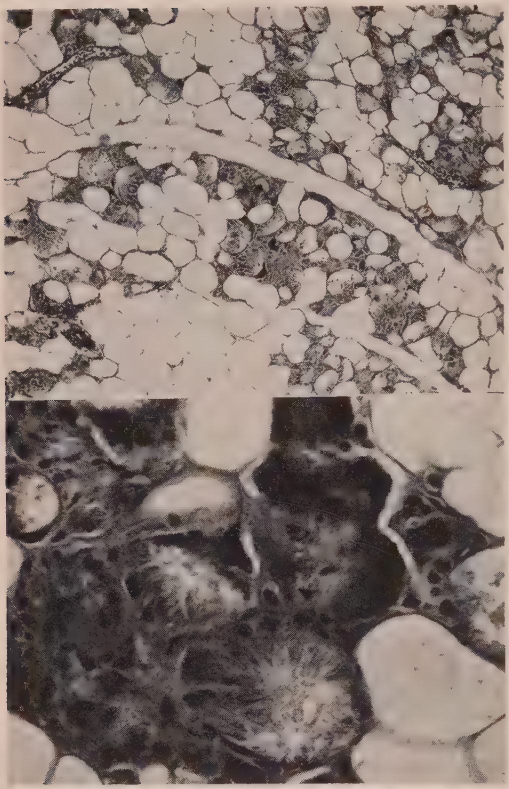

FIG. 1. *Top:* Periadrenal fat showing multiple islands of fat injury separated by normal fat. Hematoxylin and eosin, ×60. *Bottom:* Higher magnification showing variations of cellular reaction. Multinucleated giant cells are present at top of print. A fat cell containing radial slit-like spaces is seen near bottom of print. Hematoxylin and eosin, ×430.

This reaction in visceral and retroperitoneal fat of the rat bears some histologic resemblance to pathologic conditions of human fat such as fat necrosis of newborn(2), sclerema neonatorum(3), and traumatic fat necrosis of various types(4,5,6). Further studies are underway to determine the histochemical characteristics of the lesion.

Appearance of the foreign-body type reaction in all groups except that fed lard indicates that the reaction is a response to a relative or absolute excess of saturated long chain fatty acids. We are testing diets containing various proportions of saturated to unsaturated fatty acid to determine the critical ratio which produces this reaction.

Summary. A foreign-body type reaction has been observed in fat from rats fed diets containing high levels of saturated fat. The phenomenon and the dietary conditions under which it was produced, are described.

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Effect of Dilantin Sodium on Tensile Strength of Healing Wounds.* (24040)

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Hyperplasia of the oral gingiva is a common reaction among epileptics following systemic administration of sodium diphenylhydantoinate (Dilantin Sodium). The phenomenon does not occur in all patients and, in those affected, there is a wide range in severity of the lesion(1,2). The reason for this selective involvement of gingival tissues, other tissues throughout the body apparently being unaffected, is unknown nor is the mechanism of hyperplasia understood. Tissue reaction is basically a fibrous hyperplasia with formation of increased bulk of dense collagen fibers. This has suggested to Shapiro that the compound might favorably influence the healing of gingival wounds through enhancing collagenization(3). He reported that administration of dilantin to non-epileptic patients in-

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* This investigation was supported by a research grant from Nat. Inst. of Dental Research, P.H.S.

creased rate of healing of gingival wounds as judged histologically by accelerated epithelialization and increased connective tissue activity. In a related study by Forscher and Cecil, rats receiving varying doses of dilantin showed a significant decrease in acid-soluble phosphorus fraction of tissue from the palate in area that had been subjected to a radiofrequency current to induce acute inflammatory response(4). Of other chemical constituents studied, tyrosine, proline, hydroxyproline hexosamine, ribonucleic acid, desoxyribonucleic acid, inorganic phosphorus and residue phosphorus showed no significant differences from controls. Attempts have been made to produce this hyperplasia experimentally in small laboratory animals but these have been generally unsuccessful(5,6). King has reported its occurrence in the ferret(7). Many clinical observations of "dilantin gingival hyperplasia" and the report of Shapiro have indicated that further studies of the effect of this drug on connective tissue were important. For this reason, a controlled experimental investigation of the effect of dilantin sodium on tensile strength of healing wounds has been carried out.

Methods. The present study was divided into 3 parts differing basically only in dosage and manner of administration of the drug. Sixty-day-old male albino rats of Sprague-Dawley strain were used, housed in wire bottom cages and maintained on stock laboratory diet and distilled water *ad libitum*. Series I consisted initially of 24 animals equally divided into control and experimental groups. Experimental animals differed from controls only in that each animal in former group received 50 mg of dilantin sodium/day by stomach tube. Series II consisted of 36 animals divided equally into control and experimental groups. In this case the dilantin sodium was administered at 25 mg/day/animal by dissolving in a special diluent† and injecting intramuscularly. Series III was identical with Series II except that 48 animals were used and duplicate determinations were recorded. This last series was repeated for confirmation of data. In all series, dilantin sodium was administered for 18 days before induction of experimental wound. The wound was inflicted after removing hair on abdomen by a depilatory with animals under Nembutal anesthesia. A longitudinal incision was made by scalpel approximately 30 mm long and 6 mm from midline. The incision was carried through the skin and down to the fascia overlying abdominal muscles. The wounds in Series I and II were not sutured, but in Series III were closed by 2 interrupted sutures of 4/0 black silk. Duration of wound in Series I, from time of incision to sacrifice of animal, was 3 days; in Series II and III 4 days. The tensile strength of wounds was tested similar to the method described by Enzinger and Warner(8) and many others and consisted of first removing a strip of tissue, approximately 40 mm long and 10 mm wide, at right angles to original incision. Only those animals whose wounds were healing by primary intention were utilized. One end of the tissue strip was attached to a small spring clamp while a small paper cup was suspended from the other end of the tissue strip by another clamp. Bromoform, used because of its relatively high specific gravity, was then added to the cup by burette until the strip separated at line of incision. This endpoint was definite in all cases. Values were recorded as ml of bromoform required to cause separation of the wound. Histologic studies were also carried out on sections taken through the wounds of 11 animals in control group and 10 animals in experimental group of Series III. Sections were prepared in usual manner and a trichrome stain was applied as well as routine H and E.

Results. Series I group was seriously depleted by deaths due to accidental aspiration of the drug during stomach tubing and by discarding of those animals whose wounds were not healing by primary intention. Only 5 control and 4 experimental animals remained and from these 8 and 7 tensile strength determinations respectively were completed.

The data (Table I) indicate a dramatic in-

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† This diluent was furnished through the courtesy of Parke, Davis, & Co., Detroit, Mich. and consisted of 40% propylene glycol and 10% alcohol in water with sodium hydroxide to adjust to pH 12.

TABLE I. Tensile Strength of Wounds in Control and Dilantin Treated Rats.

	No. of animals	No. of determinations	ml bromoform	"t" value
		Series I		
Control	5	8	13.8 ± 2.1*	4.14
Dilantin	4	7	34.1 ±11.2	
		Series II		
Control	9	9	17.0 ± 9.3	5.48
Dilantin	12	12	38.5 ± 9.0	
		Series III		
Control	15	29	18.4 ± 7.8	4.30
Dilantin	11	21	32.4 ± 8.6	

* Mean and stand. dev.

crease in tensile strength of wounds in dilantin treated rats. The mean value, expressed as ml of bromoform is approximately 147% greater than that of control animals. While the number of animals and of determinations in this series is not large, this difference between control and experimental groups is statistically significant at the > 1% level of confidence.

Series II contained a larger number of animals at beginning of the study and of these, 9 control animals and 12 in experimental group were suitable for use. The data are very similar to those in Series I, the same dramatic difference between control and dilantin treated rats being found. Mean value in experimental group was significantly greater than the mean in control group at > 1% level and represents an increase of 127% in mean tensile strength of experimental wounds. Control studies with the diluent alone showed that it did not increase tensile strength of wounds.

The results of Series III are nearly identical with those of Series I and II. In this portion of the investigation, 15 control and 11 experimental animals remained at termination of study. The mean tensile strength of wounds in the experimental group showed a 76% increase which is significant at > 1% level.

It was not possible to differentiate between histologic sections of wounds from control and experimental animals. The trichrome stain enhanced visualization of the new collagen in the healing wounds but there was enough variation within each group to preclude using the sections as confirmatory evidence for the differences in tensile strength.

Discussion. Since tensile strength of a healing wound parallels and is dependent primarily upon collagenization of the wound, the finding of increased tensile strength of wounds in rats given dilantin sodium is consistent with the fibrous hyperplasia of the oral gingiva which is of common clinical occurrence. Although connective tissue overgrowth clinically involves only the gingiva, the drug is obviously not anatomically specific for this site. The common appearance of hyperplasia here can probably be related to the nearly universal occurrence of at least a mild chronic inflammation in tissues surrounding necks of teeth although the nature of this relationship is speculative.

Summary. Systemic administration of dilantin sodium produces a dramatic increase in tensile strength of healing wounds in rats. This is probably a function of increased collagenization, the same basic reaction which accounts for the undesirable side-reaction of gingival hyperplasia in epileptics receiving this drug, although the mechanism is unknown.

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Partial Purification of Urinary Erythropoietin.* (24041)

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Erythropoietic stimulating activity has been detected in urines of anemic rabbits(1) and some human subjects with low hemoglobin levels (below 6 g%)(2-6). Urinary erythropoietic substance(s) when administered to rats may evoke increases in total numbers of circulating erythrocytes(6) as well as in total circulating red cell volume(5). Reticulocytosis(2,5,6), marrow erythroid hyperplasia (2,6) and enhanced incorporation of Fe^{59} into the circulating erythrocytes(4) have also been observed. A shorter assay, involving reticulocyte and hematocrit determinations, was employed in this study to expedite screening of large numbers of chemical fractions. A significant increase in reticulocyte percentages unaccompanied by fall in hematocrit after 5 days treatment constituted a positive indication of presence of erythropoietic stimulating activity. From the work of Rambach *et al.* (7), and our own experiences indicating a positive correlation between toluidine blue metachromasia and presence of erythropoietic stimulating activity in plasma filtrates(8), it appeared possible that plasma erythropoietin is a mucoprotein or a moiety associated with mucoprotein. Findings of elevated mucoprotein levels in both serum of anemic rabbits (9) and in urine of rabbits rendered anemic by repeated bleedings(10) present further circumstantial support for this possibility. Urinary gonadotrophin is a substance(s) known to have chemical properties of a mucoprotein, and ultrafiltration technics for concentration of urinary gonadotrophin(11) have been recently applied(5) for concentration of urinary erythropoietic activity. We have utilized a modification of another urinary gonadotrophin procedure, namely the kaolin adsorption method(12), in an attempt to purify urinary erythropoietin.

Materials and methods. Urine for this fractionation was obtained from a 5-year-old boy with Cooley's anemia.[†] His hemoglobin level at time of urine collection was 4.5 g%. Mature Long-Evans female rats weighing 165 to 200 g (average 185 g) and maintained on a diet of Purina Chow *ad lib.* were employed for assay of filtered untreated urine. Four rats received subcutaneous injections of 1 ml daily and 4 received 3 ml daily. Reticulocyte counts were done using Brecher's method(13) prior to the injection period and on the day following fifth injections. Microcapillary hematocrit determinations were performed in duplicate at the same times. The mean increases in these 2 parameters minus mean changes in 4 untreated controls are shown in Table I.[‡] Acid washed American Standard kaolin (Fisher K-5) was used for adsorption of the urinary factor. Preliminary studies demonstrated that the amount of active material adsorbed did not vary much over pH range of 4.3 to 6.2 although pH 4.8 appeared to give slightly better results than the others. Four and a half g of kaolin was added, with stirring, to 300 ml of urine. The pH was lowered to 4.8 by addition of 2.6 ml of 1N HCl. The suspension was equilibrated for 24 hours at 5°C. The kaolin was removed by centrifugation and the supernatant fluid equilibrated with a second 4.5 g of kaolin as before. Buffers were prepared by addition of 1M acetic acid to 1M NH_4OH until solutions with pH 8, 9, and 10 were obtained. The first kaolin precipitate was then suspended, by mixing several times with a pipette, in 20 ml of cold pH 8 ammonium acetate buffer and spun

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[*] This investigation was supported by research grants from N.I.H., U.S.P.H.S.

[†] We wish to thank Dr. Harold Fink, Coney Island Hospital, Brooklyn, N. Y., for making available urine samples from this patient.

[‡] All mean control reticulocyte and hematocrit values were essentially unchanged throughout experiment. Mean reticulocyte values varied no more than .2/100 RBC and mean hematocrits varied less than 1.1% in packed cell volume.

down again. The 20 ml of supernatant fluid was placed in the freezer. This procedure was repeated twice using pH 8 buffer and then performed 3 times with each of the pH 9, pH 10, 1M NH_4OH, and 2M NH_4OH eluting solutions. Sixty ml of each eluate was therefore collected and frozen separately. The kaolin precipitate from the second equilibration was subsequently eluted twice with each buffer, first with 20 ml and then with 5 ml. This, added to the rest, yielded 85 ml of each eluate solution. The eluates were placed separately in Visking tubing and dialyzed at 5°C for 24 hours against 0.9% saline, then 24 hours against a 0.1M phosphate buffer (pH 6.8), and finally 48 hours against 0.9% saline again. All eluates were essentially neutral reacting to pH paper, reading about pH 6.8. Six groups of female rats averaging 185 to 195 g/group were used to assay the eluates. Four rats were employed in each group, and the group mean reticulocyte and hematocrit values were carefully matched before treatment. Three ml daily subcutaneous injections were given to each rat for 5 days. Reticulocyte and hematocrit values were again determined on the sixth day. The control group received similar amounts of 0.9% saline. All determinations were performed on the same days. Mean increases in peripheral parameters of treated groups minus mean changes in the control group are shown in Fig. 1.[‡] The non-dialyzable solids content of the eluates was determined by dialysis against distilled water for 36 hours and evaporation to dryness, carefully with an infra-red lamp, of 4 ml aliquots of the dialysand in aluminum weighing pans. Toluidine blue metachromasia was tested for by adding 0.05 ml of an 0.025% solution of toluidine blue O dissolved in .1M KH_2PO_4 to 0.2 ml of the solution under observation. Ultraviolet absorption spectra were taken of the eluates in a Beckman Model DU Spectrophotometer, using silica cells with 1 cm light path. An ultracentrifuge analysis was performed by Dr. Ralph Heimer on an aliquot of the 1M NH_4OH eluate concentrated 4X by dialysis against 40% polyvinyl pyrrolidone in physiological saline. This solution was spun 2 hours in a Spinco Model E analytical ultracentrifuge at 145,000 X gravity. Paper strip electrophoresis of 5 μl of the 1M eluate or of the 4X concentrate of this eluate was conducted on Whatman 3MM strips in a Spinco Model R electrophoresis cell at a constant current of 6 milliamps and 85 volts. Veronal buffer pH 8.6, ionic strength 0.075 was employed and 16 hours were allowed for migration. The strips were then dried in an oven and stained with bromphenol blue. Statistical methods used were those described by Davies(14).

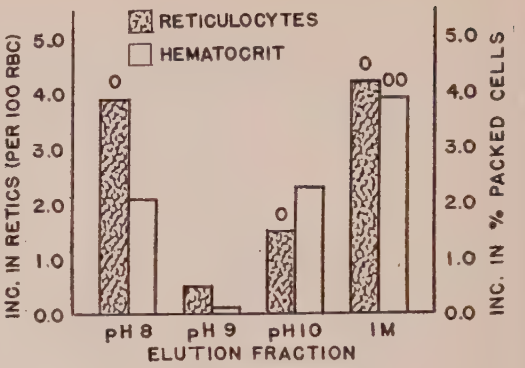

FIG. 1. Erythropoietic activity of eluates of urinary substance adsorbed on kaolin at pH 4.8. Significance of increase over pre-treatment levels, 0 = P <.01, 00 = P <.02.

TABLE I. Bioassay of Whole Anemic Human Urine.

	Response (mean increase minus control change ± S.E.)	
Dose, ml/day	Reticulocytes/100 RBC	Hematocrit, % packed cells
1	1.1 ± .5[*]	.7 ± .9
3	3.1 ± .9[†]	2.0 ± 1.1

[*] Increase over pre-treatment level—P <.05.
[†] *Idem* —P <.01.

Results. The activity recovered in different eluates from kaolin precipitates can be seen in Fig. 1. The principal active fractions were those obtained by elution at pH 8 and by elution with 1M NH_4OH. The 2M NH_4OH eluate evoked a decrease in hematocrit, hence its activity could not be determined. Since proportionality between dose of urine and reticulocyte response appears to be linear in the concentration ranges employed (Table I) it may be calculated that 36% of the original activity was recovered at pH 8, 5% at pH 9, 14% at pH 10, and 39% with 1M NH_4OH. The total recovery for all fractions was thus

TABLE II. Purity of Urinary Erythropoietin Eluted from Kaolin.

Fraction	Solid content, mg/ml	% relative activity/mg	Purification
Original urine	39.50	2.5	1
Idem (dialyzed)	1.70	59.0	24×
pH 8 eluate	3.40	37.1	15×
1M NH_4OH eluate	0.23	587.0	230×

94%. The % relative activity was obtained by dividing reticulocyte response to 3 ml daily injections of a given fraction (Fig. 1) by the response to 3 ml daily injections of starting material (Table I) and multiplying by 100. When this figure is divided by the solid content of the solution, the % relative activity/mg is obtained. Dividing % relative activity/mg of a given fraction by that of starting material provided an index of purification (Table II). Thus, dialysis alone produced a 24-fold purification while the 1M eluate represented a 230-fold purification or approximately a 10-fold purification of the non-dialyzable material.

The height of the absorption peak at 280 mμ roughly paralleled the erythropoietic activity of the fractions (Fig. 2). The most potent fraction, the 1M NH_4OH eluate absorbed considerable light at this wave length. This fraction also produced a pronounced metachromatic reaction with toluidine blue. No metachromasia was produced by the original urine or by fractions eluted with the less basic buffers. When concentrated 4 times by dialysis against 40% polyvinyl pyrrolidone and subjected to a relative centrifugal force of 145,000 X g for 2 hours there was very little displacement of the boundary, and no sharp peaks appeared. The electrophoretic pattern obtained from 5 μl of the 1M eluate revealed very little migration of a weakly staining spot near the origin of the paper strip.

Discussion. The use of kaolin for adsorption of urinary erythropoietin has enabled recovery in the 1M NH_4OH eluate of 39% of the original activity at a purity 230 times that of raw urine. This purified preparation produced a strong metachromatic reaction with toluidine blue. A similar correlation has been reported(8) between metachromasia and erythropoietic stimulating activity in boiled plasma filtrates from anemic rabbits. Failure of the original urine and the pH 8 eluate to display metachromasia may have resulted from the presence of metachromasia inhibitors. Indeed, we have found that heparin added to anemic human urine is incapable of displaying its usual pink metachromatic reaction. Presence of metachromatic properties and an absorption peak at 280 mμ in our purest fraction is not inconsistent with the suggestion that erythropoietin may be a mucoprotein or an associated substance(7). However, urinary erythropoietin does not possess any material that migrates in the paper electrophoretic system as far as alpha globulins found in anemic rabbit plasma(7). The position on the paper strip of our preparation is more like that assumed by Marcotte-Boy's fraction X(10).

Others(15-17) have suggested that erythropoietin may be a lipid and this possibility cannot be precluded. However, unlike the plasma factor of Linman and Bethell(15), urinary erythropoietin can evoke significant increases in hematocrit(6) or in circulating red cell volume(5). It would be of interest to test the combined effects of these 2 preparations.

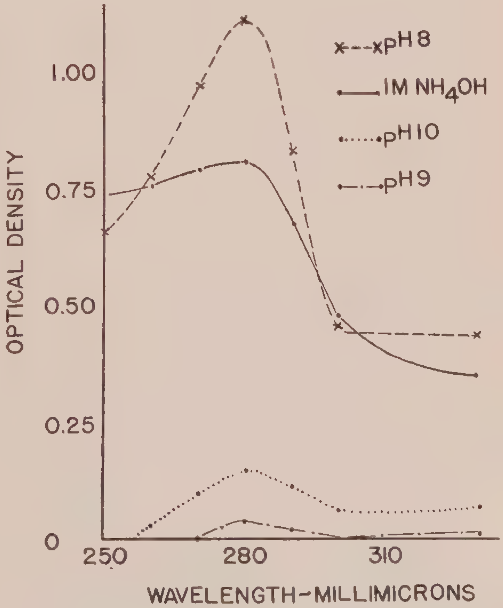

FIG. 2. Ultraviolet absorption spectra of the eluates.

Work is in progress to determine the smallest biologically active moiety in urine, its chemical nature, and whether its primary site of action is bone marrow or some other tissue or organ.

Summary. Urinary erythropoietic factor has been partially purified by adsorption on kaolin followed by serial elution with ammonium acetate buffers of increasing pH and then with 1M NH_4OH. Using this procedure, a 39% yield with a 230 fold purification of the active factor has been obtained. The purified preparation of the active factor produces metachromasia with toluidine blue, has an absorption peak at 280 mμ, and migrates very slowly on paper strips in an electrophoretic apparatus. The similarity and differences between urinary and plasma erythropoietin are discussed.

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Influence of Cortisone, Medrol, and Aristocort Diacetate on Antitryptic Activity of Rat Serum. (24042)

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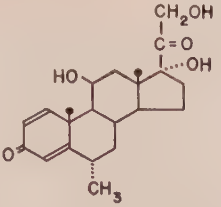
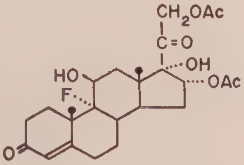
The view has been expressed that an intimate physiological relationship exists within the circulating blood between fibrin formation and dissolution (fibrinolytic) mechanisms and the integrity of the blood vessel wall(1,2). Previous work(3,4) has presented evidence that the pituitary-adrenal cortex axis exerts a controlling influence on the fibrinolytic (plasmin-antiplasmin) system in the blood. In continuation of studies(5) on the influence of certain modified corticosteroids, more potent than cortisone, on antitryptic activity of rat serum, Medrol (Δ^1-6-α methylcortisol) and Aristocort diacetate (16α-hydroxy-9α-fluorocortisol-16α, 21 diacetate) were compared with cortisone in normal, hypophysectomized and adrenalectomized rats.

Materials and methods. Male Sprague-Dawley rats weighing 150-200 g were used. Normal and hypophysectomized animals were allowed Purina Laboratory chow and water *ad libitum,* while adrenalectomized rats were fed Purina Laboratory chow and 0.9% saline *ad libitum.* All administrations were given intramuscularly; controls received either no injections, or a volume of 0.9% saline equal to that used in the steroid injections. Adrenalectomy and hypophysectomy were performed under ether anesthesia. Administration of the steroids was started 3 weeks after the operation. Suspensions of the following steroids were made by trituration and subsequent shaking with 0.9% saline to concentrations of 500 μg/ml and 2.0 mg/ml respectively: Cortisone, Medrol, and Aristocort diacetate.* The chemical structure and alternate nomenclature of the 2 latter steroids is listed in Fig. 1. The animals were given intramuscular injections of 1 μg or 4 μg per g of body weight at 24 hours intervals in the thigh muscles of either the left or right hind leg. These injections were continued for a period of 3 days. Blood samples of 2.0 ml were taken by heart puncture 72 hours and 10 days after the initial injection. Those animals assayed at 72 hours were injected at 0, 24, and 48 hours while in the 10 days study, the animals were injected at 0, 24 and 48 hours and kept 7 days before assay. The blood samples were allowed to clot at room temperature, centrifuged after 1-3 hours and the sera kept frozen until tested. Five or more animals were studied at each dosage level in the experimental groups. *Test for antitryptic activity.* The details of this procedure have been described(5,6). Antiproteolytic units refer to antitryptic activity of the experimental serum samples in terms of a "standard normal serum preparation" to which an arbitrary value of 100 antitryptic units per ml was assigned (5,6). All assays were performed in duplicate.



MEDROL = Δ'-6 α-METHYLCORTISOL
(6 α-METHYL PREDNISOLONE)



TRIAMCINOLONE ACETATE (ARISTOCORT DIACETATE)
16 α-HYDROXY-9 α-FLUOROCORTISOL-
16 α, 21-DIACETATE

FIG. 1. Structure of steroids investigated.

Results. The influence of the different corticosteroids at varying dosage levels on antitryptic activity of blood in the experimental groups is illustrated in Fig. 2. Each bar represents an average of 5 or more animals. Baseline values were obtained by averaging the saline and uninjected controls. This appeared justified since no differences in antitryptic activity were observed between these 2 groups. The baseline value represents an average of about 20 animals. It was found that the antitryptic activity returned to the preinjection level upon discontinuing the steroid administration (10 days values). The serum antitryptic values of the adrenalectomized animals were below those of the normal controls, while hypophysectomy produced baseline values lower than those found after adrenalectomy (Fig. 2). In the analysis of the effect of the steroids on antitryptic serum activity, each of the experimental groups will be considered separately.

Unoperated animals. Indication of increased antitryptic activity was noted upon cortisone (1 μg/g body weight) administration, with only a slight increase when the dosage level was increased to 4 μg/g body weight. Medrol-induced responses at 1 μg were equivalent to cortisone administration at the higher dosage level. The increased antitryptic activity at 4 μg falls within the highly significant range of $p<0.01$. Aristocort diacetate produced the most marked elevation in serum antitryptic activity; increasing the dose level to 4 μg produced no further elevation.

Hypophysectomized rats. Cortisone administration at 1 μg/g body weight produced no change, but an elevation in antitryptic activity was noted at the higher dosage level. Similarly, Medrol-induced changes were apparent

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* Cortisone was kindly supplied by Merck and Co., Rahway, N. J.; Medrol by The Upjohn Co., Kalamazoo, Mich., Aristocort diacetate by the American Cyanamid Co., Pearl River, N. Y.

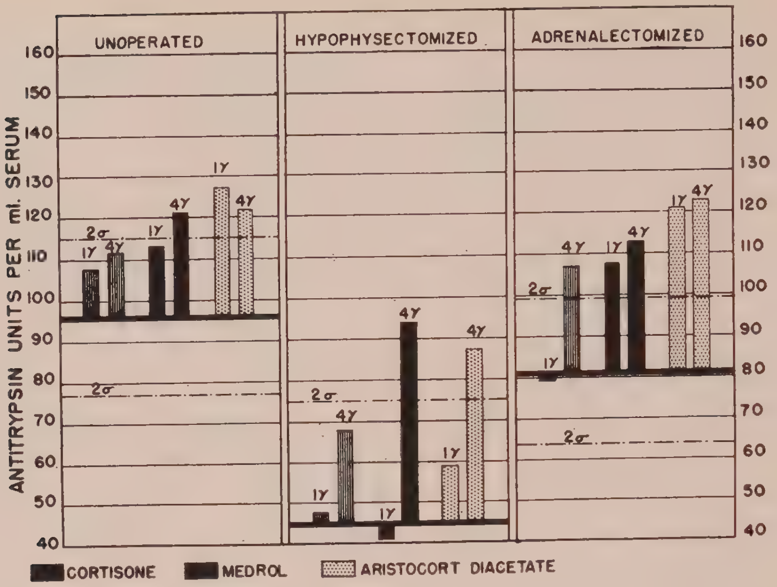

FIG. 2. Alterations in antitryptic activity 72 hr after steroid administration.

only at the higher dosage. Aristocort diacetate administration resulted in a slight increase in serum antitryptic activity at 1 μg, and in a substantial elevation when 4 μg were administered.

Adrenalectomized rats. At 1 μg/g body weight, cortisone administration produced no change in antitryptic serum activity. When a dosage level of 4 μg/g body weight was employed, a pronounced rise in antitryptic activity was evident. With Medrol administration, the increased antitryptic activity noted at a level of 1 μg was equivalent to cortisone induced changes at 4 μg/g body weight. Increasing the dosage level to 4 μg resulted in only a slight elevation over that observed at the lower level. The most pronounced elevations were noted with Aristocort diacetate administration where both dosage levels produced similar increases in antitryptic activity.

Discussion. As in the previous studies(4, 5), it was noted that adrenalectomy is associated with a lowering of the antitryptic activity (Fig. 2). Hypophysectomy produced the lowest level, which is in agreement with earlier work(4). This pronounced decrease, definitely below that observed after adrenalectomy, would tend to indicate that control of serum antitryptic activity is not solely an adrenocortical function, but that other endocrine systems are probably also involved. The data indicate that Aristocort diacetate produced the most consistent elevation in serum antitryptic activity. Medrol appeared to be next in order of effectiveness while cortisone-induced changes were apparent mainly at the higher dosage levels. No attempt was made to equate the dosage of these compounds on a molecular weight basis. It has been shown that Medrol(7) and Aristocort diacetate(8) are several times more active than cortisone with regard to glycogen deposition and anti-inflammatory activity. The great effectiveness of Medrol and Aristocort diacetate in promoting increased serum antitryptic activity suggests that these steroids may be useful in management of clinical hemorrhagic conditions associated with fibrinolysis.

Summary. The influence of cortisone, Medrol (Δ^1-6α-methylcortisol), and Aristocort diacetate (16α-hydroxy-9α-fluorocortisol-16α, 21 diacetate) administration on serum antitryptic activity, at either 1 or 4 μg/g body weight, was studied in normal, hypophysectomized and adrenalectomized rats. The most consistent elevations of antitryptic activity were demonstrated with Aristocort diacetate administration. Medrol proved to be quite effective, while cortisone induced changes were apparent only at the higher dosage levels.

The low serum antitryptic activity observed after hypophysectomy when compared with adrenalectomy would indicate that in addition to the pituitary-adrenal cortex axis other endocrine systems may be involved in the physiological control of the fibrinolytic system.

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Increased Avidity of Antibody for Baltimore 1957 Strains of Asian Influenza Virus (P-Q-R Variation)* (24043)

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Influenza A viruses isolated in Asia during early stages of the 1957 pandemic appeared to have poor antigenic potency. Serum antibody titers of patients convalescent from Asian influenza were lower than anticipated (1), and considerable doubt was cast on the efficacy of the new vaccines because they seemingly failed to induce significant antibody responses. Alternatively, these findings could be explained by insensitivity of viral antigens used to measure antibody titers. It had previously been shown that antigenically related influenza A′ viruses often differed markedly in their capacity to combine with specific antibody, a phenomenon known as the P-Q-R variation(3,4). That Asian influenza viruses might undergo the same transformation was implied by Meyer, *et al.*(1) who noted that "certain of the early passage egg fluids of strain A-Japan-305-57 were not readily inhibited by homologous immune serum and appeared to be in Q phase." This supposition is confirmed by serologic studies herein reported, which demonstrate that influenza viruses isolated from patients in Baltimore during autumn of 1957 were in the R phase, although indistinguishable antigenically from the prototype Asian influenza strain A-Japan-305-57.

Materials and methods. Viruses. The prototype Asian strain of influenza A virus, A-Japan-305-57, was obtained from Walter Reed Army Medical Research Institute as lyophilized allantoic fluid of 5th consecutive egg passage. Eight additional strains of virus used were isolated in Oct. 1957 from patients at Johns Hopkins Hospital and are designated by convention as A-Baltimore-1001-57 to A-Baltimore-1008-57. Six of these viruses were recovered from throat washings of patients with clinical manifestations of acute influenza and the remaining 2 from lung tissue of patients who died several days after onset of illness. Penicillin and streptomycin were added to the throat washings or 10% suspensions of lung tissue and 0.1 ml of each material was inoculated into amniotic cavity of chicken embryos 8 days of age. After incubation for an additional 5 days at 37°C the embryos were chilled, amniotic fluids harvested and the presence of virus determined by capacity of fluids to agglutinate an equal volume of 1% guinea pig erythrocytes. Each virus isolate was passaged an additional 1-3 times by the allantoic route in 10 or 11-day-old chicken

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* Supported by grant from Nat. Cancer Inst., U.S.P.H.S., and contract with U. S. Army Chemical Corps, Fort Detrick, Md.

embryos and the final virus suspension was stored at –60°C prior to use. A-Japan-305-57 virus was studied after it had undergone a total of 9 allantoic passages. *Immune sera.* The Communicable Disease Center of the U.S. Public Health Service kindly supplied 2 standard preparations of chicken antiserum, anti-A-Japan-305-57 and anti-A-Denver-1-57 (a recent A′ strain). Additional immune sera were prepared in this laboratory by intraperitoneal injection of chickens with A-Japan-305-57 or A-Baltimore-1001-57 viruses. Each chicken received approximately 320-400 CCA doses of a virus suspension on each of 3 successive days and was bled 8 days after last injection. The Maryland State Dept. of Health generously furnished paired serum specimens from men vaccinated with commercial monovalent Asian influenza vaccine. Paired sera were also obtained from patients admitted to Johns Hopkins Hospital with clinical diagnosis of influenza. Sera from vaccinated individuals were received after they had been inactivated at 56°C for 30 min.; all other human and chicken sera were unheated and stored in frozen state without addition of preservatives. None of the sera was treated with cholera filtrate or periodate. *Hemagglutination titrations* were performed by modification of the Salk(2) pattern test, in which the endpoint was read as partial (50%) agglutination of 0.5% chicken erythrocytes in final volume of 0.5 ml. In the *hemagglutination-inhibition (HI) tests* serial 2-fold dilutions of serum were made in 0.25 ml of 1% chicken erythrocytes and 5 agglutinating doses of virus were added to each tube to give final volume of 0.5 ml. The tests were read after incubation at 4°C for 30 minutes (which gave the clearest pattern). HI titers are expressed as original dilution of serum producing partial (50%) inhibition of hemagglutination. *Neutralization tests* were performed in chicken embryos with 1% normal and immune chicken sera heated at 56°C for 30 minutes. Higher concentrations of immune serum completely neutralized infectivity of one of the strains. Serial 10-fold dilutions of virus were made in normal or immune serum, or broth-saline solution. After incubation of the virus-serum mixtures for 1 hour at room temperature, they were inoculated by allantoic route into groups of 6 eggs. Allantoic fluids were harvested 44 hours later, and the presence of virus determined by agglutination of 1% chicken erythrocytes. The 50% egg infective dose (EID_{50}) was calculated by the method of Reed and Muench.

TABLE I. HI Antibody Titers of C.D.C. Reference Chicken Antisera.

		Antisera	
Virus strain	Source	A-Japan-305-57 (Asian)	A-Denver-1-57 (A′)
A-Japan- 305-57	Prototype	80*	<10
A-Balt. -1001- ”	Throat	640	<10
-1002- ”	”	160	”
-1003- ”	Lung	240	< 5
-1004- ”	Throat	160	<10
-1005- ”	Lung	”	”
-1006- ”	Throat	320	10
-1007- ”	”	”	<10
-1008- ”	”	160	20

* Reciprocal.

Results. Serologic identification of Baltimore viruses. The 8 strains of virus isolated during these studies were identified as Asian serotypes by HI tests performed with reference C.D.C. chicken antisera (Table I). As noted, anti-A-Japan-305-57 antiserum inhibited hemagglutinating activity of all 8 Baltimore viruses at dilutions of 1:160 or greater, whereas antiserum prepared against the A′ Denver strain gave HI titers of 1:20 or less. Non-specific inhibitors rather than antibody may have accounted for the slight inhibitory effect of anti-A-Denver-1-57 serum against 2 of the Baltimore viruses. However, the most striking finding was that anti-A-Japan-305-57 antiserum was invariably a more effective inhibitor of the Baltimore viruses than of the homologous strain.

Cross hemagglutination - inhibition tests with Japanese and Baltimore viruses and their respective antisera. Further studies were carried out to characterize the antigenic relationships of viruses isolated in Japan and Baltimore and to determine their relative capacities to stimulate antibody production. Three chickens were immunized with an allantoic fluid suspension of A-Japan-305-57 virus and 3 others with A-Baltimore-1001-57 virus, each

TABLE II. HI Titers of Antisera from Chickens Immunized with Equivalent Amounts of Japanese or Baltimore Viruses.

	Antiserum					
	Anti-A-Japan-305-57			Anti-A-Balt.-1001-57		
Virus strain	C81*	C82	C83	C84	C85	C86
A-Japan- 305-57	60†	160	160	120	240	60
A-Balt. -1001- "	1920	640	1280	2560	1920	2560
-1003- "	640	1280	480	960	2560	1920

* C81 = Chicken No. 81.
† Reciprocal of serum dilution.

of the 6 animals receiving equivalent amounts of viral hemagglutinin on the same dosage schedule. Sera prepared from immunized chickens were tested individually for their HI antibody content against Japanese and 2 Baltimore viruses. Table II demonstrates that high titers of HI antibody were produced against both strains of Baltimore virus, regardless of whether chickens were immunized with Japanese or Baltimore virus. Similarly, both types of antisera inhibited A-Japan-305-57 virus to the same degree. It appears, therefore, that Japanese and Baltimore viruses have similar antigenic structures and equivalent immunizing potency in chickens. However, it is readily apparent that the hemagglutinating activity of A-Japan-305-57 virus was invariably less well inhibited by either homologous or heterologous antisera than were hemagglutinins of the Baltimore viruses. Therefore, the Japanese strain was in the Q phase, differing serologically from 2 Baltimore strains only in its relative insusceptibility to antibody.

Increased susceptibility of Baltimore virus to neutralization by immune serum. Infectivity of Baltimore virus for chicken embryos was more readily neutralized by anti-A-Japan-305-57 serum than was the infectivity of the homologous virus. The titers of A-Japan-305-57 virus diluted in broth-saline solution, 1% normal chicken serum and 1% immune serum were $10^{7.5}$, $10^{6.3}$, and $10^{5.2}$ EID_{50}, respectively. Corresponding determinations of infectivity of Baltimore-1003-57 virus diluted in broth-saline solution, normal serum and immune serum were $10^{5.7}$, $10^{5.9}$, and $10^{2.2}$ EID_{50}, respectively. Therefore, the neutralization index for A-Japan-305-57 virus, calculated on basis of its infectivity in 1% normal and immune chicken serum, was 12, whereas neutralization index for A-Baltimore-1003-57 virus was 5000. Thus, the Baltimore virus, compared with the prototype Japanese strain, exhibits greater sensitivity to neutralization of infectivity, as well as greater sensitivity to inhibition of hemagglutination by immune serum.

Paradoxical serologic response of individuals vaccinated with A-Japan-305-57 virus. Paired sera were obtained from men immunized with a single intradermal injection of 0.1 ml of formalinized vaccine prepared from A-Japan-305-57 virus. This vaccination program had been instituted the month prior to onset of the Baltimore epidemic. It is highly unlikely, therefore, that these individuals had been exposed to active infection with Asian influenza virus before post-vaccination sera were obtained. The antibody content of serum collected on the day of vaccination and 14-22 days later was determined by HI tests using antigens prepared from A-Japan-305-57 and 2 strains of Baltimore virus. Representative data presented in Table III demonstrate that no antibodies could be detected against the homologous strain of virus. Slight antihemagglutinating activity of pre-vaccination sera against the Baltimore viruses can be attributed to nonspecific inhibitor. Notwithstanding, each vaccinated individual showed a definite rise in antibody titer against the heterologous viruses. It is evident, therefore, that HI tests with R phase Baltimore viruses are more sensitive indicators of immunologic response than are tests with the prototype

TABLE III. HI Antibody Response to Intradermal Vaccination with A-Japan-305-57 Virus.

	Human sera					
Virus strain	No. 30244 Pre*	No. 30244 Post*	No. 30563 Pre	No. 30563 Post	No. 30566 Pre	No. 30566 Post
A-Japan-305-57	0†	0	0	0	0	0
A-Balt.-						
1001-57	0	80	10	80	20	80
1003-57	0	20	30	160	40	160

* Pre- or post-vaccination.
† 0 = <5 (reciprocal).

TABLE IV. HI Antibody Response to Infection during the 1957 Baltimore Epidemic of Influenza.

	Patients' sera							
	P. McC.		S. W.		H. B.		E. P.	
Virus strain	Acute	Conv.	Acute	Conv.	Acute	Conv.	Acute	Conv.
A-Japan-305-57	<10*	80	<10	40	<40	80	<10	<10
A-Balt.-1003-57	30	320	"	80	"	320	"	40

* Reciprocal of serum dilution.

Asian strain in the Q phase.

HI antibody response to infection. Serum was obtained from unvaccinated patients within 48 hours of onset of influenza and 2-3 weeks later. These paired sera were tested against both A-Japan-305-57 and A-Baltimore-1003-57 viruses. Representative data on 4 patients are shown in Table IV to illustrate that serum antihemagglutinin titers in convalescence were invariably higher when A-Baltimore-1003-57 virus was used as test antigen. It should also be noted that patient E.P., from whose throat washings we failed to isolate a virus, would have been undiagnosed if serologic studies had been done with A-Japan-305-57 virus alone.

Discussion. The term "P-Q-R variation" was coined by van der Veen and Mulder(3) to designate differences in avidity of antibody for influenza viruses of the same antigenic type. Viruses that are poorly inhibited by homologous and heterologous antiserum are said to be in the Q phase, as opposed to P phase viruses which are inhibited to high titer by homologous but not by heterologous antiserum. A virus in the R phase is inhibited to high titer by antiserum prepared from P, Q or R strains. Therefore, HI antibody determinations performed with Q phase virus as antigen will give fortuitously low titers. Isaacs and Andrewes(4,5) used these biological marker characteristics to great advantage in studying variants of A′ influenza virus responsible for the epidemics of 1950-1951. In their studies Q phase viruses predominated in early outbreaks and were gradually replaced by P phase viruses, although both forms were sometimes present in a population at the same time. They also demonstrated that P-Q transformations could be reproduced in the laboratory by infecting animals under conditions which simulate those in human epidemics. P phase variants of A-Sweden-3-50 (Q) virus emerged after passage in the lungs of non-immune mice. Conversely, P phase virus was gradually converted to a Q form by passage in chicken embryos in the presence of subinhibitory doses of homologous antiserum. These authors also demonstrated that the P-Q-R variation is not an all-or-none phenomenon, but that a spectrum of strains exists with intermediate susceptibility to antibody.

The present studies demonstrate that in Oct. 1957 Baltimore strains of Asian influenza virus were isolated in the R phase in contrast to the Q phase A-Japan-305-57 virus isolated 7 months previously. Chickens immunized with the Japanese strain invariably developed higher HI and neutralizing antibody titers when serum was tested with Baltimore viruses rather than with the homologous strain. In fact, intradermal vaccination of human subjects with A-Japan-305-57 virus would have been considered totally inadequate if HI antibody response had been measured with the homologous (Q) strain alone. Serologic studies of patients convalescent from influenza were far easier to interpret when carried out with antibody-sensitive Baltimore (R) viruses. Nevertheless, the Baltimore strains of virus were indistinguishable antigenically from the prototype Asian strain as determined by cross hemagglutination-inhibition tests with chicken antisera. Although it has been suggested(3,4,5) that P and R phase viruses are more effective immunizing agents, similar HI titers were produced in chickens immunized with Asian strains in either the Q or R phase.

Summary. Asian influenza A viruses isolated during the Baltimore epidemic in Oct. 1957 were in the R phase of the P-Q-R variation although indistinguishable antigenically

from the Q phase prototype virus A-Japan-305-57.

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Pineal and Hypophyseal Phosphate Uptake After Castration and Administration of Sex Hormones.* (24044)

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Physiological roles attributed to the pineal organ in terrestrial vertebrates have usually involved gonads and hypophysis, but relationships to the control of blood pressure and general body activity have been suggested also (1-10). Unfortunately, many results are contradictory and are not supported by quantitative or statistical documentation. The suggestive and significant studies of Borell and Orström(11-15) on pineal phosphate uptake or turnover merit reexamination. Their finding that rat pineal has a turnover rate 3 to 4 times that of "adenohypophysis" and much greater than that of various parts of brain has been confirmed by Reiss, Badrick and Halkerston(16). Although incorporation of P^{32}-labeled phosphate into pineal phospholipids and proteins as well as into carbohydrate phosphate esters occurred within 40 minutes of intraperitoneal injection(12), the major portion of P32 uptake can justifiably still be considered as an indicator of metabolic rate, as it is generally in other organs. Among interesting results obtained by Borell and Orström (11-13) with female rats are: (1) increase in phosphate turnover in hypophysis and tuber cinereum and decrease in ovary and uterus after pinealectomy (operated at 50-100 g; sacrificed 5 weeks to 9 months later), and (2) increase in hypophyseal and pineal phosphate turnover 6 weeks after castration (6 ♂'s and 6 ♀'s). Apparently, tissue samples were combined to facilitate P determinations and no standard errors are provided. They postulated that pineal activates ovaries and conversely is inhibited by them(13). This conclusion contradicts the view held by other workers that the pineal is primarily antigonadal or antihypophysis(10).

Materials and methods. Female rats of Long-Evans and 2 mixed strains were used. In most experiments, litter-mate experimental and control animals were used in pairs and handled in identical manners. All animals received Diablo Double Check Labration pellets and tap water *ad lib.* Animal room temperature was 75-80° F. and artificial illumination was provided in single daily photo-periods 12-14 hours. Bilaterally ovariectomized rats and their sham-operated controls were maintained 3 weeks to 2 months (Table I) before autopsy. Daily subcutaneous injections of 17β-estradiol (Schering) were administered in 0.2 ml sesame oil during 4 days preceding autopsy. Injections of progesterone ("Proluton," Schering) were administered similarly in daily doses of 0.1 ml (5 mg) during 3 days preceding autopsy. Control animals were injected similarly with equivalent volumes of

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* This investigation was supported by research grants from National Science Foundation, Nat. Inst. of Health, P.H.S., and Cancer Research Funds of Univ. of California. We are grateful to Dr. Preston L. Perlman of Schering Corp., for hormones, to Depts. of Genetics and Psychology for some animals used, to Drs. Morgan Harris, H. A. Bern, and K. B. DeOme for facilities and to Jean Grant and Tom Fashinell for technical aid.

TABLE I. Influence of Castration and Sex Hormones on Relative (P^{32}) Specific Activity in Pineal and Ant. and Post. Hypophysis in Female Rats (Long-Evans *(LE)*, *Mixed*, and Dept. of Genetics Hybrid *(Gen)* Strains). Autopsy 30 min. after intraper. inj. of P^{32} phosphate.

Treatment (strains)	Age at operation autopsy (days)		P^{32} dose, μc	No. of animals	Ant. hypophysis		Post. hypophysis		Pineal		Ratios	
	operation	autopsy			x	± S.E.	x	± S.E.	x	± S.E.	P/AP	P/PP
Ovariectomy:	30–53	87–98	75	14–15	6.78	.89	12.53	1.79	37.10	5.45	5.5	3.0
sham operated				13–14	6.91	1.14	10.07	1.64	33.08	5.48	4.8	3.3
(LE)												
OVARIECTOMY + OIL:												
Ovariectomy +												
2 μg estradiol	25–36	50–61	75	6	*5.13	.78	9.53	1.30	34.86	7.24	6.8	3.7
(LE)				5	*7.94	.63	9.41	.54	36.55	5.18	4.6	3.9
2 μg estradiol	23–38	62–76	87	5	7.50	1.40	14.49	3.33	54.72	14.60	7.3	3.8
(Gen)				5	8.77	1.40	15.00	2.13	62.32	8.07	7.1	4.1
Totals of above				11	6.32	.85	12.01	1.88	44.79	8.36	7.1	3.7
				10	8.32	.69	11.95	1.30	48.26	5.97	5.8	4.0
Ovariectomy +												
5 mg progesterone	18–23	58–63	75	8–9	4.99	.47	10.08	1.14	37.35	4.40	7.5	3.7
(LE)				6–7	5.21	.86	10.89	1.61	46.33	9.95	8.9	4.3
Ovariectomy +												
250 μg estradiol	32,	56,	75–95	11	†4.01	.51	9.02	.90	33.26	3.28	8.3	3.7
(mixed)	43–51	79–93		11	†9.03	.95	11.53	1.26	40.59	4.06	4.5	3.5
INTACT + OIL:												
Intact +												
250 μg estradiol		59–71	75	12	7.29	1.35	13.20	2.56	42.75	8.53	5.9	3.2
(mixed)				11	9.77	.99	12.61	1.56	44.73	7.46	4.6	3.5
Intact +												
250 μg estradiol		200–230	93	5–6	11.60	1.32	18.33	1.88	66.16	4.97	5.7	3.6
(Gen)				6	16.63	2.08	23.00	2.50	81.55	5.31	4.9	3.5
250 μg estradiol		old	75	6	6.14	.86	10.38	1.59	28.13	2.91	4.6	2.7
(LE)				6	5.33	.74	7.16	.93	24.75	2.75	4.6	3.5

x = mean; S.E. = stand. error of mean.

* $P < 0.02$ } (Student-Fisher t test, for significance of differences).
† $P < 0.001$ }

sesame oil. During injection periods, control and experimental animals were kept in separate cages, usually in pairs. Methods of measuring phosphorus turnover are essentially those employed by Borell and Orström(11-15) but we made determinations for individual animals rather than grouped samples. Thirty to 40 minutes before autopsy each animal was injected intraperitoneally with 0.5 ml of isotonic saline containing 20-95 μc P^{32} as sodium phosphate. Autopsy was performed following ether anesthesia. Body weights and vaginal smears were recorded for each animal. The pineal, anterior lobe, posterior lobe (pars intermedia and pars nervosa) of the hypophysis, anterior part of cerebellar vermis, and a small drop of blood were placed separately on small pieces of ash-free filter paper, squashed and dried overnight at 37°C. Tissues were individually counted about 24 hours after autopsy in a flow counter. About a month later, total phosphate determinations were made utilizing King's modification of the Fiske-Subbarow technic(17) with "Elon" (methyl P-amino-phenol sulfate) as reducing agent and heating of samples (Gomori, 18). Readings were made on Beckman Model DU spectrophotometer. In the first experiment, counts/min/mg dry weight were obtained for each tissue sample. In other experiments "specific activity" (counts/min/total μg P) and "relative specific activity" (specific activity of tissue/specific activity of cerebellum of same animal) were obtained.

Results. The effect of bilateral ovariectomy on phosphorus turnover in the adult female was examined first, using tissue weight, and comparing relative specific activity with respect to blood and cerebellum. No differences were found. From data of other experiments (Table I) it may be seen that in relation to ovariectomy and treatment with sex hormones: (1) post. hypophysis is unaffected, (2) ant. hypophysis shows increased uptake after treatment with estradiol, especially in castrate and after pharmacological doses, (3) pineal, while in general slightly more active after steroid hormone treatment, does not seem to be *significantly* modified by any one treatment, (4) relative specific activity of the pineal is 4.5 to 8.9 times that of the ant. hypophysis and 2.7 to 4.3 times that of post. hypophysis.

Discussion. According to Borell, Westman and Orström(19) relative specific activities of the rat "adenohypophysis" and tuber cinereum are greatest during proestrus. In later studies(20) it was shown that only 5000 I.B.U. or more estradiol injected daily for 3 days was followed in 48 hours by increased relative specific activity in the adenohypophysis of intact female rats (80-145 g). In our experiments also, increased ant. hypophyseal phosphate uptake occurred most clearly after pharmacological doses of estradiol. Relative specific activity of "adenohypophysis" in rabbits was found by Borell, Westman and Orström(21) to be similar at estrus and anestrus and was increased 3 to 5 weeks after castration and following coitus. Likewise, increase in "adenohypophyseal" phosphate uptake was noted by Borell and Orström(13) in the rat (6 ♂, 6 ♀) "at least 6 weeks" after castration. But we have been unsuccessful in producing an unequivocal increase in ant. pituitary activity after castration (Table I). The variables of age and laboratory strain of rat may possibly be responsible for this discrepancy.

Mechanisms for regulation of pituitary metabolism and gonadotropic activity and their responses to castration and sex hormones have been extensively studied in the past and are closely dependent on age of animals, dosage and timing. It must be thought, moreover, that physiological modification of hypophyseal gonadotropic activity need not be necessarily reflected in a detectable change in metabolic rate or phosphate turnover of ant. hypophysis as a whole.

We have utilized recent work on effects of ovariectomy, estradiol and progesterone on gonadotropin secretion in the rat to provide procedures that are capable of modifying FSH and LH secretion in treated animals. If a major portion of pineal parenchyma is metabolically responsive to female sex hormones or gonadotropin levels, changes in rate of pineal phosphate uptake would be expected. However, in our experiments such changes marked enough to be regarded as significant, did not occur.

The ratio of pineal relative specific activity to relative specific activity of ant. hypophysis (Table I) shows wide fluctuation without apparent correlation with experimental treatment, whereas the post. hypophysis ratio varies less.

Summary. Uptake of radiophosphate (P^{32}) in the pineal and ant. and post. hypophysis was studied in female rats after ovariectomy and daily treatment with 17β-estradiol (2 or 250 μg) or progesterone (5 mg). Significant change (increase) in uptake occurred only in the ant. hypophysis after estradiol. Relative specific activity (in relation to cerebellar vermis) of the pineal is 4.5-8.9 times that of the anterior lobe and 2.7-4.3 times that of the posterior lobe of the pituitary gland and remains high in adult and old animals.

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Effect of Antioxidants on Muscle and Plasma Lipids of Vit. E-deficient Guinea Pigs.*† (24045)

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Evidence has been accumulating showing that addition of certain antioxidants to Vit. E-deficient diets either delays or prevents occurrence of Vit. E-deficiency lesions in skeletal muscle of animals. DPPD (N, N′-diphenyl-p-phenylenediamine) delays the onset of muscular dystrophy in lambs fed a Vit. E-deficient diet(1) and also prevents muscular degeneration in chicks fed diets low in both Vit. E and sulfur(2). DPPD, DBH (2,5-di-*t* - butylhydroquinone) and Santoquin (6-ethoxy-2,2,4-trimethyl - 1,2-dihydroquinoline) delay but do not prevent occurrence of muscular dystrophy in guinea pigs fed a highly purified diet deficient in Vit. E(3). Accumulation of cholesterol and total lipid in the skeletal muscle in animals deficient in Vit. E (4-9) and the hypercholesteremia observed in Vit. E-deficient rabbits(4) suggests that this vitamin may have a role in controlling lipid metabolism of skeletal muscle and plasma. Since certain antioxidants can replace Vit. E in one of its functions, it seemed desirable to determine whether the ability of Vit. E to function as antioxidant is responsible for its role in regulation of cholesterol and lipid metabolism. Accordingly an investigation was undertaken in which groups of guinea pigs were fed diets free of Vit. E and diets supplemented with either α-tocopherol acetate or various other antioxidants. Progress of guinea pigs was closely followed to determine incidence and frequency of dystrophy.

Procedure. Female guinea pigs were fed a purified diet consisting of casein, 30 g; cornstarch, 19.5 g; dextrose, 11 g; sucrose, 10 g; cellulose, 10 g; Wesson salt mix, 6 g; stripped lard, 5 g; agar, 5 g; potassium acetate, 2.5 g; magnesium oxide, 0.5 g; inositol, 0.2 g; choline chloride, 0.2 g; niacin, 20 mg; p-amino benzoic acid, 10 mg; calcium pantothenate, 4 mg; thiamine chloride, 1.6 mg; riboflavin, 1.6 mg; pyridoxine hydrochloride, 1.6 mg; folic acid, 1 mg; 2-methyl-1,4-naphthoquinone, 0.5 mg; biotin, 0.1 mg; Vit. B_{12}, 10 μg; Vit. A, 500 U.S.P. units and Vit. D_2, 50 U.S.P. units; the tocopherol content of this diet is less than 25 μg. In addition, each animal received daily (6 times/week) an oral

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TABLE I. Cholesterol Content of Blood Plasma.

Dietary supplement	Condition of animals	No. of animals	Plasma cholesterol (mg %)	
			Free	Total
α-tocopherol acetate	Normal	3	17.6 ± 1.12*	48.5 ± 1.40*
None	Non-dystrophic	3	34.2 ± 13.9	75.1 ± 15.4
”	Dystrophic	3	57.9 ± 10.7	119 ± 20.4
BHT	Non-dystrophic	5	25.7 ± 7.03	68.8 ± 7.71
Santoquin	”	6	24.1 ± 3.68	77.0 ± 8.90
DPPD	”	6	24.9 ± 2.73	91.3 ± 9.65
DBH	”	6	33.6 ± 12.9	91.1 ± 10.8

* ± S.E.

supplement of 10 mg ascorbic acid in 0.1 ml water. One group of guinea pigs received this basal diet, a second group received the basal diet plus additional oral supplement of α-tocopherol acetate (5 mg/day, 6 times/week) and each of the remaining groups received one of the following antioxidants at a level of 0.025% of the diet: DPPD, Santoquin, BHT (butylated hydroxytoluene) or DBH. After 6 months of feeding, non-dystrophic animals from each group were autopsied; in these animals the creatine content of the gastrocnemius muscle as determined by the Jaffe reaction(10,11) was not depressed. The remaining animals in each group were maintained on experimental rations until they became dystrophic; at this time they were autopsied. All animals were sacrificed under Nembutal anesthesia. Blood samples were obtained by heart puncture. Individual bloods were centrifuged and the lipids extracted from the plasmas with ethanol-acetone (1:1). Skeletal muscle was removed from thigh, ground with acid-washed sand and anhydrous sodium sulfate and then extracted with boiling ethanol-petroleum ether (skellysolve B) (3:20). The supernatant was decanted, and the residue was extracted twice more with fresh portions of boiling petroleum ether, which were added to the first extract. All extracts were analyzed for free and total cholesterol by the method of Nieft and Deuel (12). Total lipids were determined gravimetrically on petroleum ether extracts of muscle.

Results. A marked increase obtains in both free and total cholesterol levels of plasma (Table I) of dystrophic animals fed the diet deficient in Vit. E as compared with animals fed the complete diet. This is in agreement with our previous results in Vit. E-deficient guinea pigs and rabbits(6) and with findings of Morgulis and Spencer in Vit. E-deficient rabbits(4). In animals fed the Vit. E-deficient diet and sacrificed before onset of paralysis in what may be considered an early stage of the disease, there was also evidence of an increased plasma cholesterol concentration although less marked than in dystrophic animals fed the Vit. E-deficient ration. These elevations of plasma cholesterol are similar to those observed in the remaining groups where Vit. E-deficient diet has been supplemented with antioxidants BHT, Santoquin, DPPD and DBH.

Elevation of skeletal muscle cholesterol (Table II) in dystrophic animals fed the Vit. E-deficient diet also agrees with earlier findings(4-9); these increases also precede the onset of dystrophy. Both prior to dystrophy and after paralysis a similar increase obtains in cholesterol of skeletal muscle of guinea pigs receiving BHT, Santoquin, DPPD, and DBH.

The muscle total lipid levels (Table II) of all groups except the dystrophic animals receiving DBH are elevated over those of animals receiving Vit. E; moreover, the levels of all groups receiving antioxidants are below those of the animals receiving the Vit. E-deficient diet. However, due to the great variation in these values within the groups, it is not possible to conclude that antioxidants prevent the rise of muscle total lipid in Vit. E-deficiency.‡

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‡ It is possible that the slightly beneficial effects of antioxidants other than Vit. E, which have been observed, may be due in part to the fact that these antioxidants spare traces of Vit. E in the diet.

TABLE II. Free and Total Cholesterol and Total Lipid Content of Skeletal Muscle.

Dietary supplement	Condition of animals	No. of animals	Cholesterol, mg/g wet wt		Total lipid, g/100 g wet wt
			Free	Total	
α-tocopherol acetate	Non-dystrophic	6	.66 ± .05*	.72 ± .06*	2.13 ± .02*
None	Non-dystrophic	7	1.07 ± .05	1.16 ± .06	4.11 ± .24
	Dystrophic	7	1.39 ± .13	1.44 ± .13	5.10 ±1.12
BHT	Non-dystrophic	5	1.01 ± .04	1.05 ± .04	3.84 ± .49
	Dystrophic	3	1.37 ± .25	1.40 ± .26	3.13 ± .58
Santoquin	Non-dystrophic	6	.98 ± .16	.98 ± .14	2.69 ± .90
	Dystrophic	4	.87 ± .05	.90 ± .06	2.68 ± .38
DPPD	Non-dystrophic	6	1.11 ± .07	1.13 ± .07	3.66 ± .54
	Dystrophic	3	1.03 ± .11	1.01 ± .13	4.05 ±1.20
DBH	Non-dystrophic	6	1.15 ± .05	1.17 ± .04	2.89 ± .18
	Dystrophic	3	1.64 ± .14	1.72 ± .11	1.98 ± .11

* ± S.E.

These results indicate that the effect of Vit. E on cholesterol and lipid levels of skeletal muscle and plasma is not that of a nonspecific *in vivo* lipid antioxidant as evidenced by inability of antioxidants tested to maintain normal cholesterol and total lipid levels in the absence of Vit. E. Vit. E probably exerts its effect on cholesterol and lipid metabolism through some enzymatic mechanism in which non-tocopherol antioxidants are inactive; in this connection, it is of interest that the tocopherols but not DPPD and Santoquin stimulate isooctane-extracted, digitonin-solubilized DPN-cytochrome c reductase(13).

Summary. In guinea pigs with muscular dystrophy induced by feeding of Vit. E-deficient ration, there is a rise in cholesterol levels of plasma and skeletal muscle; these rises precede onset of dystrophy. Supplementation of Vit. E-deficient ration with antioxidants DPPD, BHT, DBH and Santoquin, although delaying onset of dystrophy symptoms, does not prevent the rise in plasma and muscle cholesterol and muscle total lipid in Vit. E-deficiency.

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Convenient Method for Assay *in Vivo* of Antimetabolites of Serotonin (24046)

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Existing methods for measurement of the potency of antagonists of serotonin employ either isolated segments of smooth muscle or intact animals in which can be observed a more or less specific response to injection of the hormone. Thus, segments of carotid arteries(1) or portions of the uterus taken from an estrogenized rat(2-4) contract when treated with serotonin, and this response can be overcome with suitable antagonists. In this way an *in vitro* assay can be made to measure the potency of such antagonists. *In vivo* measurements can be made by determination of amount of an antagonist which will prevent pressor effects of serotonin injected into a dog(3,5) or to prevent antidiuretic effect of the hormone injected into hydrated rats(6). The *in vitro* assays suffer from the very serious handicap that many antiserotonins which prove to be quite active on the isolated tissues fail to show any significant potency when tested on whole animals(3,5). The *in vivo* assays, such as the one involving measurement of effects on blood pressure, are time-consuming. At least a week of intensive work may be necessary in order to establish the potency of a single compound. Consequently, a more convenient *in vivo* assay may be desirable. The method here described depends on the fact that when a mouse is exposed to a continuous source of serotonin a very severe diarrhea ensues. This can be prevented if the animal has been treated previously with an antimetabolite of the hormone. The continuous source of serotonin is provided by injection of 5-hydroxytryptophane. Udenfriend *et al.*(7) have shown that administration of this amino acid to rats brings about a large increase in serotonin content of most tissues. Because the hydroxytryptophane is converted to serotonin continuously for several hours the increased content of this hormone is maintained in tissues for a relatively long time. This is in contrast to what follows the injection of a single dose of serotonin itself. The difference can be seen readily when one measures increase in blood pressure, or diarrhea. After injection of serotonin an animal frequently defecates because of strong contractions of the intestinal muscles which can be easily observed. The effect is transient however, so that all individuals do not defecate, and those which do, do so only for a very short time. With hydroxytryptophane, however, the contractions continue for a long time. The result is that all individuals exhibit profound diarrhea lasting for a long time. Woolley *et al.*(8) showed that the diarrhea which follows injection of 5-hydroxytryptophane could be completely prevented by prior treatment of animals with a potent antimetabolite of serotonin. This observation has now been made the basis of a method for assay of such antimetabolites in living animals.

Materials. The antimetabolites of serotonin were synthesized in the ways described previously(9-12). The 5-hydroxytryptophane was the racemic form, and was generously supplied by Abbott Laboratories and by the National Drug Co. All compounds were dissolved in Ringer's solution and adjusted to pH 7.5 before injection.

Method. Adult female Swiss mice weighing 25 to 28 g which had been caged for at least one week together in groups of 6 were placed individually in 1 liter beakers for 30 minutes prior to the assay. Any which exhibited diarrhea during this control period were discarded. A group of 6 animals was selected for each dose level of each of the compounds to be examined. Each mouse was then injected intraperitoneally with 0.5 ml of Ringer's solution containing the desired amount of compound to be assayed. Graded doses of each compound were chosen to cover the range of 0 mg up to an amount which would protect all 6 mice in the group. A group was also included which received no antimetabolite and no hydroxytryptophane but just Ringer's solution.

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* With technical assistance of M. Gallagher and S. Kaufman.

Thirty minutes after injection of the antiserotonin each mouse received 1 mg of DL-5-hydroxytryptophane in 0.25 ml of Ringer's solution injected intraperitoneally. The animals were then observed very closely for 30 minutes. The onset of diarrhea, and the severity of it were recorded from visual observation of the excreta in each beaker during this period. It was necessary to keep constant watch of all animals. Mere examination of the beakers at the end of the 30 min. period was not sufficient because if this was done evaporation from watery feces and cleaning of the beakers by the mice tended to obscure the extent of the diarrhea. Severe watery diarrhea of persistent character was seen in all individuals which had received only the hydroxytryptophane. This usually began about 10 min. after injection of the hydroxytryptophane. In those which had been given in addition an amount of an antiserotonin sufficient to confer partial protection, severe diarrhea was seen in some, mild diarrhea in others, and no diarrhea in the remainder. The mild diarrhea might be only the ejection of a few watery droppings from time to time after the passage of some solid feces or it might be one short episode of watery droppings. It was easily distinguished from the severe and prolonged type. The mere passage of normal feces, even though in increased amount, was not counted as diarrhea. Typical responses of mice so treated with one potent antiserotonin are shown in Table I. This compound was 1-benzyl-2-methyl-5-methoxytryptamine hydrochloride, the "benzyl analog of serotonin" or BAS(5).

TABLE I. Diarrhea in Mice Given BAS and 5-Hydroxytryptophane. All mice received 1 mg DL-5-hydroxytryptophane 30 min. after the BAS. BAS = 1-benzyl-2-methyl-5-methoxytryptamine hydrochloride.

BAS, mg/kg	No. of mice	Severe diarrhea	Mild diarrhea
0	6	6	0
4	6	6	0
6	6	5	0
8	6	3	2
10	6	2	1
12	6	2	1
15	6	1	2
20	6	0	0

The quantity of an antiserotonin such as BAS required to protect half of the mice was determined by plotting a dose-response curve from data such as those shown in Table I. For this purpose the number of mice in each group which showed severe diarrhea was plotted against the dosage of antiserotonin. Each mouse with mild diarrhea was counted as 0.5 of a severe-diarrhea-mouse. The amount of BAS required to protect half of the mice did not vary greatly from day to day over a period of 8 months, although there was some variation. Actual values found in 8 experiments were 8.0, 8.5, 9.0, 15.0, 8.0, 12.5, 12.0, and 10.4 mg BAS per kg.

In order to compare the potencies of 2 compounds each was assayed at the same time in the manner described. In the present work BAS was used as standard. The results of such experiments with several compounds are shown in Table II. The amount of an antiserotonin required to protect half of the animals could, of course, be determined more accurately than that needed to protect all, but the usefulness for other purposes of the latter quantity made it advisable to determine and record it as well. It was also found desirable to include in the assay of any antiserotonin dosages ranging from that giving complete protection to that giving none.

The relative potencies of some of the antimetabolites of Table II were known from experiments in dogs in which the ability of each compound to protect against the pressor action of intravenous serotonin had been measured. These relative potencies are included to indicate that although the species and the organ system involved were different, the 2 types of assay rated the compounds in the same order. It is, of course, to be expected that the correlation will not be exact. In fact, it is already known(10) that the route of administration of the anti-metabolites (whether orally or intravenously) can markedly alter the potency in the dog test. However, comparison of results found by intravenous ad-

TABLE II. Amounts of Various Antimetabolites of Serotonin Required to Protect Mice against the Diarrhea Induced by 5-Hydroxytryptophane.

Antimetabolites	Source	Dose for complete protection, mg/kg	Dose for 50% protection, mg/kg	Relative potency in dogs, mg/mg of BAS-phenol
BAS = 1-benzyl-2-methyl-5-methoxytryptamine hydrochloride	(9)	20	10	8
BAS-phenol = 1-benzyl-2-methyl-5-hydroxytryptamine hydrochloride	(10)	8	2	1
BAB = 1-benzyl-2-methyl-5-methoxy-N,N-dimethyltryptamine hydrochloride	(9,11)	32	8	3
3,4,5-Trimethoxyphenoxyethylamine hydrochloride	(12)	40	20	
2,6-Dimethoxyphenoxyethylamine hydrochloride*	(12)	40	22	

* This compound by itself with no hydroxytryptophane caused diarrhea in some mice at 10-20 mg/kg. This may reflect its serotonin-like action on certain isolated tissues(12).

ministration to dogs shown in Table II with those by intraperitoneal administration in mice may indicate that the new method may be of some value in attempts to find useful antiserotonins.

Great precision is not claimed for the present assay method. It might possibly be improved by use of larger numbers of animals in each dosage group, or by other means. The objection which we have found to increase in the numbers is that when more mice are employed the observer's time is so occupied in watching the large numbers that the behavior of individuals is neglected with consequent loss in accuracy. The present procedure seems sufficiently precise to rank antiserotonins in order of activity which is enough for present needs.

Because the dose of 5-hydroxytryptophane used was very large, one might think that the assay could be rendered more sensitive by reduction of this dose. The dose was set so high in order to insure that all of the controls would exhibit diarrhea, and thus simplify the statistical problem which would otherwise arise. Among several hundred mice given the amount of hydroxytryptophane recommended (1 mg) none has failed to show diarrhea. It is true that with the present method rather large amounts of an antiserotonin are needed to protect the animals. This is however of some advantage in attempts to find highly potent antagonists of serotonin because the doubtful cases of compounds with low potency tend to be eliminated. Despite these considerations many experiments were done with smaller doses of hydroxytryptophane and with BAS as the antagonist. These showed that with 0.2 mg of hydroxytryptophane per mouse (still enough to cause diarrhea in all) instead of 1 mg the same quantity of BAS was required as with the larger dose. This fact was probably related to the irreversible character of the antagonism of BAS to serotonin(5). Once a tissue has been protected with this antagonist it will not readily respond even to increased amounts of serotonin. Possibly with reversible antiserotonins a reduction in the challenging dose of hydroxytryptophane might make the assay more sensitive.

The design of the assay made possible experiments on the duration of an antiserotonin effect in an animal. Increases in the time interval between injection of antimetabolite and hydroxytryptophane changed the amount of drug required to give protection of half of the mice, and thus permitted study of this property of persistence of each compound. Because various compounds are excreted (or inactivated) at differing rates, care was taken to observe the same time intervals in all assays, unless persistence was being investigated.

The reason for the control period of time at the start of the assay (the 30 minutes before any compound was injected) was the elimination of animals which will show diarrhea without any antimetabolite or hydroxytryptophane. Unless such mice were eliminated the results were discordant. In order to minimize the number showing such spontaneous diarrhea it was found helpful to cage the animals together in groups of 6 for about a week so that they were not under the stress of being subjected to a new social order immediately before the assay was performed. Because of the possibility of spontaneous diarrhea a control group receiving no drug and no hydroxytryptophane is recommended. This spontaneous diarrhea was not due to salmonellosis or other infections but seemed to occur among a few individuals even though they were in good health.

Summary. An assay for the potency of antimetabolites of serotonin has been described. This assay was rapid and easy to perform and the results were readily reproducible. The recommended method was an *in vivo* one which measured the effects on the intestinal tract. It involved determination of the potency of a compound in causing protection against diarrhea when a large dose of the serotonin-precursor, 5 - hydroxytryptophane, was injected.

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Nucleic Acids and Their Derivatives in Alcoholic Extracts of Rat Tumors and Normal Muscle.* (24047)

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Our object has been to study the chemistry of a highly selected fraction of tumor tissue *i.e.*, an alcoholic extract, because such a tumor extract appeared to have unique biological properties when injected into living tumors, and, as we showed, it also elicited immunological responses of interest in tumor immunology. Earlier studies showed that there were marked differences between substances extracted with alcohol from tumors and from host muscle tissue. For example, in tumor extracts inorganic phosphorus, glucose, lactic acid and ribonucleic acid (RNA) were low, whereas the deoxyribonucleic acid (DNA) was 3-fold high(1). Differences were found also between amino acid composition (2), as well as between protein contents(3) of tumor extracts, and similar extracts made from muscle. The immunology and chemistry of these proteins were studied(3). The present study is limited to investigation of DNA and RNA components which are different in the 2 types of extracts.

Materials and methods. Three sarcomas (Aptekman sarcoma No. 6 and Lewis sarcomas Nos. 7 and 10) originally induced in albino-inbred rats† by injections of methylcholanthrene and then carried on by transplantation, were used. These tumors were of the late generation spindle-cell type following induction by hydrocarbons, described by

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† Rats bearing these tumors were kindly supplied by Dr. Margaret R. Lewis of Wistar Institute.

Lewis(4). Two batches of extracts were also made from a lymphoma‡ No. 8 found by Lewis(5). It had arisen spontaneously in the submaxillary gland of a female breeder of the Lewis inbred strain of rats. *Extracts* were made as previously described(2) in the cold with final concentration of 80% alcohol. They were clarified by filtration after concentration to 30% alcohol at 30°C and standing at –15°C. Since no adequate control is available for tumor tissue, the host tissue, muscle, was chosen for comparison. Similar extracts were, therefore, made from muscle of these same rats and from normal rats, and rats whose tumors had been made to slough off following injection of tumor extracts and which were then shown to be resistant to further challenge with tumor fragments. *Analyses* of the extracts were made for content of ash free solids, RNA or ribosides by orcinol reaction of Drury(6) modified by Miller, Golder and Miller(7), and DNA or deoxyribosides by the diphenylamine reaction of Dische(8), modified for quantitative use by Seibert(9). Sensitivity in the latter method was increased, as suggested by Patterson and Dackerman (10), by heating 24 hours at 37°C instead of boiling 15 minutes. Deoxyribosides were also determined by the tryptophane perchloric acid method, described by Cohen(11) and adapted for use by Seibert, Pfaff and Seibert(12). *Ultraviolet absorption* measurements were made in Beckman spectrophotometer, model DU, on solutions and paper strips which had been subjected to electrophoresis. The strips were passed through attachment designed by Tennent for the Beckman spectrophotometer and described by Block, Durrum and Zweig (13). *Electrophoresis* experiments were carried out in paper strip model apparatus. Four half-inch strips were run simultaneously for 2 hours using phosphate buffer pH 7.38, 0.1 μ. The strips were dried in oven at 90°C for 15 minutes and then studied in 3 different ways. Some were measured in the Beckman spectrophotometer to locate position of ultraviolet absorbing components. Others were sprayed with a modified Dische (cysteine: H_2SO_4) reagent(14) for detecting presence of DNA or deoxyribonucleosides as a pink to rose color. Other strips were sprayed with 2% orcinol in 2 N-HCl, as recommended by Bidwell, Krotkov and Reed(15). A blue color appeared in presence of ribosides (guanosine and adenosine) and a faint one for RNA. *Microbiological assay* for total free deoxyribosides in tumor extracts was also made by the method‡ recommended by Hoff-Jørgenson (16), using the bacterium, *Thermobacterium acidophilus R 26,* which is highly dependent upon presence of these substances. The medium was that recommended by Toennies, Usdin, and Phillips(17), and pure thymidine served as standard. Quantities of extracts, determined by preliminary trial to have a content of deoxyribosides equivalent to 0.01 to 0.5 μg of thymidine/ml final medium, were mixed with equal volume of double strength basal medium and then inoculated with 0.05 ml of a suspension of the organism in its log phase of growth(18). The inoculated sample was then incubated at 37°C for 18-24 hours and turbidity determined in the Klett colorimeter, using λ 660 filter. Comparisons were made, as recommended by Toennies and Gallant(19), with results obtained on a solution of basal medium containing pure thymidine as standard. Analyses for pyrimidine deoxyribosides were also made on aliquots of the extracts which had been adjusted to pH 2.0 and then hydrolyzed in boiling water bath for 5 minutes to destroy purine deoxyribosides, as shown by Hoff-Jørgensen(16). *Thymine* and/or thymidine was also determined by means of the bacterium, *E. coli T*(–) mutant, dependent upon these substances for growth. The medium was double strength basal medium described by MacLoed(20) and Roepke, Libby and Small(21), and method of assay was similar to that described above.

Results. Spectral absorption. Total nucleic acid content of extracts was obtained by adding mg/ml of RNA, determined by orcinol reaction, and mg/ml of DNA, found by diphenylamine reaction. Specific density/mg of nucleic acid was obtained by dividing ultra-

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‡ We are deeply grateful to Miss Travaglini, Lankenau Research Institute, for demonstrating the significance of these methods, for supplying cultures and assistance and advice in technics.

TABLE I. Specific Density Related to Nucleic Acid Content of Extracts.

Extracts	No. of extracts studied	Nucleic acid calculated as RNA (orcinol) mg/ml	DNA (diphenylamine) mg/ml	Specific density per mg of total nucleic acid
Tumor*	7	2.1	.11	27.5 (20.9–35.7)†
Muscles (normal)	4	3.7	.04	15.8 (15.4–16.2)
" (tumor-bearing)	5	4.0	.06	17.7 (15.4–19.8)
" (immune)	3	3.8	.04	16.8 (15.9–17.3)
Dialysates of				
Tumor		2.1	.11	35.9
Muscle		10.0	.18	22.5
DNA				19.5
RNA				20.4
Guanosine				42.6
Adenosine				46.0
Uridine				43.0
Deoxyguanosine				50.0
Deoxyadenosine				58.0
Thymidine				44.3

* Results on extracts from sarcomas No. 6 (2), No. 7 (1), No. 10 (2) and lymphoma No. 8 (2) were averaged.

† Range in parentheses.

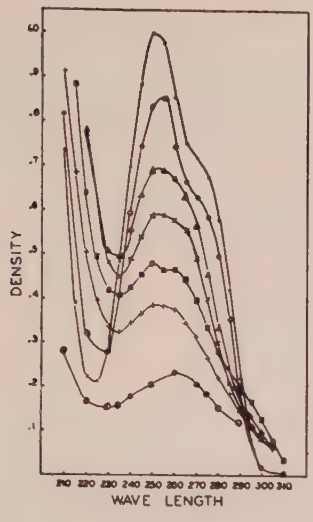
violet absorption density/ml at maximum wave length (λ 2500 or λ 2600) by content of total RNA plus DNA/ml. Table I shows that the specific density of 7 tumor extracts (20.9 to 35.7) was consistently higher than that for 12 extracts of muscle tissue (15.1 to 19.8), and also higher than for pure DNA or RNA (19.5 and 20.4). Moreover, the highly-absorbing substance was dialyzable.

Since ribosides and deoxyribosides have very high specific absorptions with maxima at the same wave length (λ 2500) as tumor extracts, and since the absorption for guanosine and deoxyguanosine show a bulge to the right (at λ 2600 to λ 2700), similar to that for tumor extracts (Fig. 1 a), the possibility of their presence in tumor extracts was considered. In contrast, muscle extracts, as well as adenosine, deoxyadenosine, thymidine and uridine gave single homogeneous curves (Fig. 1 b).

Electrophoresis. Four paper strips, on each of which was placed 4-10 μl of tumor extract, after subjection to electrophoresis for 2 hours, were studied as follows, (1) examination in duplicate for the position of ultraviolet-absorbing components in the special adapter for the Beckman spectrophotometer, (2) for position of blue-staining components with orcinol spray reagent, which would indicate RNA or ribosides, and (3) for position of pink-staining components with the cysteine: H_2SO_4 spray reagent for detection of presence of DNA or deoxyribosides. Pure nucleic acids, ribosides, deoxyribosides, and muscle extracts were studied in similar manner.

Pure DNA and RNA migrated about 35 mm toward the cathode, as shown by ultraviolet absorption at λ 2600 and also by the fact that a blue spot in the same position resulted from the orcinol spray in the case of RNA, and a pink spot in the case of DNA. By the same technics pure guanosine or adenosine migrated about 35 mm toward the anode, and were identified as blue-staining components with the orcinol spray, and as ultra-violet-absorbing at λ 2500 to λ 2600. Pure deoxyguanosine and deoxyadenosine also migrated about 35 mm toward the anode, but were identified as pink-staining components with cysteine: H_2SO_4 spray, and as ultraviolet-absorbing at λ 2500 to λ 2600.

Tumor extracts showed an ultraviolet-absorbing (λ 2550) component about 30 mm toward the anode, and at the same position a component stained pink with cysteine: H_2SO_4 spray, indicating presence of deoxyribosides. No blue staining component was found. Dialysate of the tumor extract gave similar results.



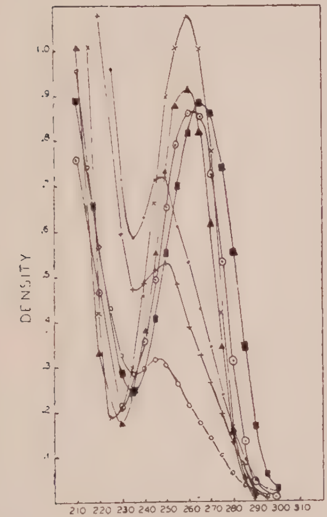


FIG. 1 a (above). Curves of spectral density at different wave lengths from top to bottom. Deoxyguanosine (●——●); guanosine (○——○); tumor extract (▲——▲); tumor extract (×——×); tumor extract (■——■); dialysate of tumor extract (+——+); DNA (⊙——⊙).

FIG. 1 b (below). Curves of spectral density at different wave lengths from top to bottom. Deoxyadenosine (×——×); adenosine (▲——▲); thymidine (■——■); uridine (⊙——⊙); normal muscle extract (●——●); normal muscle extract (+——+); dialysate of normal muscle extract (○——○).

Muscle extracts, on the other hand, showed an ultraviolet-absorbing component (λ 2550) about 30 mm toward the anode, and a blue-staining component at this position, indicating presence of ribosides. Another weakly-staining blue component appeared about 30 mm toward the cathode, probably RNA. No pink-staining material was present, except a trace toward the anode in the dialysate of the muscle extract.

Bioassay analyses. Studies of purine and pyrimidine deoxyribosides by bioassay methods revealed the fact that the ratio of these 2 types of deoxyribosides was not the same in tumor extracts as in normal muscle extracts. For example, the average ratio of purine to pyrimidine deoxyribosides in 5 tumor extracts was 1:1.8 whereas in 3 normal muscle extracts it was 1:3.0 (Table II).

That ratios on lymphoma extracts were closer to those on muscle tissue than were those on sarcoma extracts is consistent with results obtained by Miller(3), who found a protein component only in lymphoma extracts similar to one found in normal muscle extract, and suggested it was due to incorporation of some normal muscle tissue during excision of the widely dispersed lymphomas.

Chemical analyses by diphenylamine, which detects DNA and purine deoxyribosides, and by tryptophane perchloric acid method, which detects in addition pyrimidine deoxyribosides, ascorbic acid and fructose, supported to some extent the difference in ratios observed by the bioassay method. If the difference in the results calculated as deoxyguanosine by these 2 methods (Table III) is considered to be due to pyrimidine deoxyribosides, then the ratio of purine deoxyriboside to pyrimidine deoxyriboside in tumor extracts is 1:5.5 and in muscle extracts 1:10.04. Thus there was relatively about twice as much pyrimidine deoxyribosides in muscle extracts as in tumor extracts, as had been found by bioassay method. However, that all values by these methods were considerably higher than by bioassay methods indicates their lower degree of specificity and that such components as free deoxyribose, ascorbic acid, etc. may be included.

Summary. Spectral absorption analyses on alcoholic extracts of tumors revealed that they had a higher specific density/mg of nucleic acid than did similar extracts of normal muscle or than pure DNA or RNA. This high specific density, as well as type of ab-

TABLE II. Concentration of Deoxyribosides in Tumor and Normal Muscle Extracts (μg/mg Ash-free Solids).

Extracts from rat	Deoxyribosides			Thymine or thymidine	Ratios, purine to pyrimidine deoxyribosides
	Total	Pyrimidine	Purine		
Sarcoma No. 6	1.37	.73	.64	.23	
” No. 7	1.50	.92	.59	.27	
” No. 10	1.66	1.00	.65	.34	
Avg	1.51	.88	.63	.28	1:1.4
Lymphoma No. 8	4.58	2.85	1.68	2.05	
” No. 8	3.19	2.40	.79	1.80	
Avg	3.89	2.63	1.24	1.96	1:2.1
Normal muscle	.65	.50	.16	.10	
” ”	.68	.50	.18	.11	
” ”	.58	.43	.15	.06	
Avg	.64	.47	.16	.09	1:3.0

TABLE III. Content of Deoxyribosides in Tumor and Muscle Extracts by Chemical Analyses.

Extract	No. of extracts	Deoxyguanosine by— Tryptophane perchloric acid method, μ/mg	Deoxyguanosine by— Diphenylamine method, μ/mg	Pyrimidine deoxyribosides by difference, μ/mg	Ratio, purine to pyrimidine deoxyriboside
Tumor*	7	14.14	2.17	11.97	1: 5.5
Muscle (normal)	4	8.06	.73	7.33	1:10.04

* Results on sarcomas No. 6 (2), No. 7 (1), No. 10 (2) and lymphoma No. 8 (2) were averaged.

sorption curve, suggested the presence of guanosine or deoxyguanosine. Paper electrophoresis experiments, followed by study of paper strips for the position of ultraviolet-absorbing components, and for presence of ribosides by an orcinol spray, or of deoxyribosides by cysteine: H_2SO_4 spray, led to the conclusion that tumor extracts contained chiefly deoxyribosides, while muscle extract contained ribosides. The small amount of deoxyribosides in normal muscle extracts in contrast to tumor extracts was supported by chemical analyses and by highly specific bioassay methods. Moreover, by these methods the relative amounts of purine deoxyribosides and pyrimidine deoxyribosides in tumor extracts differed from those in normal muscle extracts, suggesting that different nucleic acid derivatives exist in tumor and the host muscle tissue.

I wish to express my appreciation for valuable technical assistance to Eva Soto-Figueroa, Mabel V. Seibert, Mildred Meekins and Marian Butts.

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Comparison of New Chemical Method of Determining Isonicotinoyl Hydrazide in Serum with Microbiological Assay. (24048)

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No satisfactory chemical method of determining isoniazid (INH) in blood of patients is as yet available, due to the insensitivity of present colorimetric methods. Extraction with organic solvents as proposed by Kelly and Poet(1) is difficult because of an unfavorable distribution coefficient between water and solvent. 1-chloro-2, 4-dinitrobenzene has been used by Ballard and Scott(2) for pharmaceutical assays of INH, and 1-fluoro-2, 4-dinitrobenzene by Yamaguchi(3) for determination of INH in rabbit urine for quantities far beyond the doses used clinically. No method for determination of INH in blood was mentioned. The present paper describes a spectrophotometric method for determination of INH in blood using 1-fluoro-2, 4-dinitrobenzene. Preliminary tests in aqueous medium showed that the color intensity was influenced by pH; therefore, serum deproteinization methods using either alkali or acid were not applicable.

Methods. Aliquots of 2 cc INH free serum or plasma were mixed with 1 cc absolute alcohol containing graded quantities of INH, 7 cc alcohol was added, the mixture shaken for 5 minutes in a mechanical shaker, centrifuged and 5 cc of clear supernatant (representing 1 cc serum) was used for colorimetry. After addition of 0.5 cc 0.4% alcoholic solution of reagent and approximately 0.1 g borax, the mixture was shaken, heated in screw cap-sealed tube 5 minutes at 80° and decanted from the excess borax for colorimetry. Concentrations of 0.0, 2.5, 5 and 10 μg INH in these samples (= μg/cc serum) were read at wave lengths from 450 to 520 mμ using the 0.0 μg/cc concentration as the blank. Fig. 1 shows the optical densities at various wave lengths on common coordinate paper. Higher degrees of absorption were observed at lower wave lengths, but readings, conforming to the Beer-Lambert Law were found at 500 mμ, which was used for subsequent work.

Results. Line 1, Fig. 2 shows the relation between concentration/cc serum and optical transmission by this method for quantities up to 10 μg per ml of INH. The points were averages of 10 determinations which showed very small deviations. At low INH content of the serum, the readings became less accurate. Experiments were carried out to use a double quantity of serum (4 cc) to be deproteinized with alcohol. In this case the ratio had to be 4 : 6, giving 60% alcohol dilution. Reading in this case had to be done at a temperature >60°. Line 2, Fig. 2 shows the readings for double quantities of serum and INH but is plotted for μg/cc serum. The curves obtained in 60% and 80% alcohol give

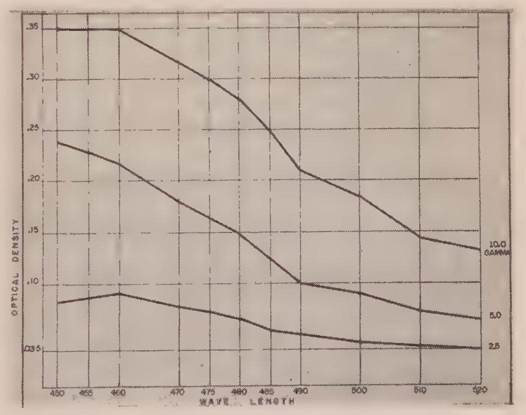

FIG. 1. Absorption spectra for 3 concentrations (μg/ml).

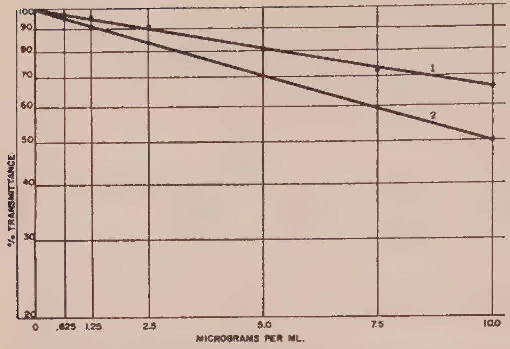

FIG. 2. Transmittance *vs* concentration ratios on semi-logarithmic paper. Serum to alcohol ratio: Line (1) 2:8; (2) 4:6.

straight lines, but have different coefficients. Recovery tests were carried out by precipitating the serum with 4 volumes of alcohol and adding to the deproteinized liquid measured amounts of INH in 80% alcohol, making allowance for the increased volume. The values of INH found when it was added after deproteinization were insignificantly higher than those obtained when the addition was made before deproteinization (Table I). INH free serum or plasma that is used in preparation of blank and standards gives a slightly yellow coloring with the fluoro reagent, so it is recommended that a pre-medication serum be used as the blank in assaying sera of elevated icterus indices. In present clinical practice, the microbiological assay of INH in serum is the accepted method for evaluation of efficacy of treatment(4). While this method measures the actual bacteriostatic efficacy of the serum, it admits a large margin of error, because of the fact that only concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 are tested (Bell and Riemensnider 5). This should be considered in the appreciation of Table II. It was not possible to obtain enough serum in every case to run both tests, and in many cases samples for chemical and biological assays were taken on different days. If both assays were performed on the same sample, the case is marked with an asterisk. The microbiological assays were carried out at Colorado Fn. for Research in Tuberculosis, Denver. Table II shows that there is sufficient agreement between the micro-biological and the chemical methods so that the latter might be used in clinical work.

TABLE I. Recovery of INH following Deproteinization.

INH added before deproteinization (μg)	Optical density	INH added after deproteinization (μg)	Optical density
5	.067	5	.071
10	.136	10	.136
10	.136	10	.138
10	.137	10	.142
20	.274	20	.283

TABLE II. Comparison of Chemical and Microbiological INH Assay.

Bioassay, μg/ml serum		Chemical assay, μg/ml	
After 2 hr	After 6 hr	After 2 hr	After 6 hr
3.2	.8	3.5	1.3
3.2	.8	3.0	1.3
.4	<.2	1.0	.17
.8	<.2	.4	.0
.8	<.2	3.6	.6
3.2	.8	3.7	1.7
1.6	.2	1.8	.18
1.6	.8	3.8	2.5
3.2	1.6	3.3	1.5
1.6	.8	3.0	.6
1.6	.8	3.7	1.6
*3.2	.8	2.0	1.3
<.2	<.2	.6	.0
1.6	1.6	1.5	1.2
1.6	.2	2.6	.9
*1.6		1.3	
3.2	1.6	2.8	.7
*3.2	.4	3.7	.4
*3.2	.8	3.0	.6
*3.2	1.6	2.9	1.7
1.6	.2	1.1	.0
1.4	.8	1.5	.9
*3.2	.8	4.0	1.6
3.2	.8	2.1	.9
*3.2	1.6	2.1	1.9
*1.6	.2	2.0	.0
* .8	<.2	1.7	.2
	.2	2.0	.7
*3.2		3.0	2.0
*3.2	1.6	3.4	1.9

Discussion. The question may be raised whether both methods determine the same chemical compounds. The microbiological method obviously determines INH and such possible metabolites as are still active as tuberculostatics. In the conditions as used in our tests, the fluoro reagent does not give reactions with isonicotinic acid and acetylisonicotinoyl hydrazide, the most prominent metabolites of INH, which both are therapeutically inert. A reactivity with the pyridine N, therefore, is not probable. Since the reagent gives a color reaction with phenylhydrazine, we are inclined to believe that the free NH_2 group of the hydrazine is essential. If there be tuberculostatic metabolites of INH present they either do not react with the reagent, or their amount is too insignificant to influence the readings noticeably. Of importance is, however, that the fluoro compound does not react with p-aminosalicylic acid, which agent is so frequently given together with INH.

The method can easily be adapted to determination of INH in urine. Since the INH concentration in urines is rather high, the urine must be diluted which usually goes to a point, when the yellow color does not interfere. At lower concentrations, the patient's urine may be matched in color with a normal urine at 500 mμ by proper dilution, then the estimation may be carried out in alcoholic or aqueous medium. In the latter case 0.5 cc 0.1N NaOH should be used as alkali. The advantages of the chemical method consist mainly in saving of time. About 8 determinations can be carried out in one hour, so that the results are available on the day when the serum has been taken, whereas the microbiological test requires several weeks.

Summary. A chemical determination of INH in patient's blood has been devised which can be carried out within one hour. The serum is deproteinized with 4 volumes of alcohol, centrifuged, and quantities corresponding to one cc serum are heated with 0.5 cc 0.4% 1-fluoro-2.4-dinitrobenzene solution in alcohol and with 0.1 g borax for 5 minutes at 80°. The developed color is read at a wave length of 500 mμ. The method gives good agreement with results obtained by the microbiological methods within the errors allowed for clinical purposes.

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Plasminogen Activator in Animal Meninges.* (24049)

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Presence of an activator of plasminogen in animal tissues was demonstrated by Astrup and Permin(1), and a method for its quantitative estimation in various organs has been published(2). With this method animal and human organs could be divided into groups of high, medium and low concentrations of plasminogen activator, although large individual variations were encountered(3,4). Lewis and Ferguson(5) found moderate fibrinolytic activity in dog meninges caused by an activator of plasminogen. Their method was not quantitative. Using the quantitative method, Roberts and Astrup(6) found large concentrations in meninges of monkeys. We have recently observed surprisingly high fibrinolytic activities of membranes of the brain(7). This observation has prompted a more thorough examination of the meninges.

Methods. Brains from freshly slaughtered, healthy animals were immediately examined or stored at –20° for later use. Dog brains were obtained from dogs used for experiments on vascular surgery (courtesy of Dr. H. H. Wandall). The Rhesus monkey brains were kindly supplied by Dr. Preben von Magnus

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TABLE I. Concentration of Plasminogen Activator in Individual Samples of Animal Meninges. Figures express units of activator/g fresh tissue. Obtained from dilution curves where each estimation is an avg of 3 determinations, and calculated by interpolation on a standard curve. Stand. dev., 7%(2). No. of animals investigated in parentheses.

Pig (11)	Ox (8)	Rabbit (4x5)	Dog (4)	Horse (3)				Monkey (4)		
Pia				Pia						
				Front lobe	Occ. lobe	Cerebellum	Chor. plex.	Pia	Dura	Sinus long. sup.
95, 110, 190	200, 300	75	810	1740	2080	1670	550	950	220	600
355, 740, 935	400, 400	150	1230	2000	3300	1320	650	1500	250	429
935, 1110, 1510	450, 470	165	2460	3020	3080		410	2250	325	420
1930, 4255	490, 540	190	4440					1235	140	500
									350 (spinal)	
									700 (cranial)	

of the Polio Department of State Serum Institute. The pia was carefully removed and freed from adhering brain tissue. No attempt was made to remove the vessels. In some animals the dura mater was similarly examined. The tissues were finely cut, and concentration of plasminogen activator estimated as described by Astrup and Albrechtsen(2). In some experiments the tissues were soaked in water for ½ hour in order to remove excess blood, and adhering water was then removed by pressing between filter paper before cutting. No significant differences between results of the 2 procedures were noticed. Activator concentration is expressed in units of the standard preparation used in previous communications from this laboratory(2,3,4,6). In rabbits the material was collected from 5 animals for each estimation which therefore represents an average value.

Results. Variations in concentration (Table I) from species to species and between individuals are in accordance with estimations in other tissues(3,4,6). Some samples from pig, dog and horse contain the highest concentrations of plasminogen activator yet recorded in any tissue. So far concentrations higher than 500 units per gram fresh tissue have only been recorded in samples from a few human organs (uterus, adrenals, lymph nodes, prostate(4)). The differences in concentrations observed in various parts of the pia could be caused by variations in the number of blood vessels. This suggestion is not necessarily contradicted by the lower activity in pia from the chorioid plexus, since the vessels here are to a great extent capillaries. This result is apparently in accordance with the observation by Astrup and Claassen(8) that fibrinolytic activity of the adventitia (of the human aorta) is high with low activity in the tunica media and no activity in the intima. Though smaller arteries have not yet been investigated it seems permissible to assume that endothelium is the part of the vessel showing least fibrinolytic activity. If this explanation also applies to the differences observed between pia, dura and the superior longitudinal sinus is unknown.

Extremely high fibrinolytic activity was also observed in human meninges. Details will be published elsewhere and their possible relation to the pathogenesis of cerebral hemorrhage discussed.

Summary. 1. Concentrations of plasminogen activator in the pia mater from pigs, horses, cows, monkeys, dogs, and rabbits were generally higher than recorded for any other organs in these animals. 2. Different pia sections of the same animal (horse) showed different activator concentrations. 3. Dura mater (monkey) had lower activity than pia. The wall of the superior longitudinal sinus had higher activity than the dura adjacent to the skull.

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Macroscopic Studies of Platelet Agglutination; Nature of Thrombocyte Agglutinating Activity of Plasma.* (24050)

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The spontaneous agglutination of platelets is poorly understood. From the earliest work(1) to the present, studies have been based primarily on microscopic observations. This report describes a test by which time and degree of agglutination are determined macroscopically. Optimum conditions for rapid agglutination of platelets are outlined.

Materials and methods. Preparation of sodium citrate, sodium oxalate, citrate-saline and saturated ammonium sulfate solutions has been described(2). Solutions of $CaCl_2$, $MgCl_2$, and $MnCl_2$ (Baker A.R.) were standardized by chloride analysis and diluted to 0.108 M. Buffered isotonic *EDTA* solution was prepared from the di- and trisodium salts of ethylenediamine tetraacetic acid (Geigy). Na_2EDTA and Na_3EDTA concentrations were 0.04086 M and 0.04734 M respectively. pH was 7.38. The solution was isosmotic with 0.156 M NaCl as determined by freezing point depression. Plasmas and platelet preparations were made from normal or hemophilic canine blood. Methods for preparing citrate plasma, oxalate plasma, fibrinogen(2), and plasmas adsorbed with $BaSO_4$ or fuller's earth (f.e.)(3) have been described. For preparation of *resin plasma,* 7 parts of blood were drawn into a syringe containing 1 part moist resin (Na cycle Dowex 50 cation exchange resin, 20-50 mesh). The blood was then passed twice through a column containing 2½ parts resin, at a flow rate of 6 ml/min. After centrifugation (2500 *g*, 20 min), the plasma was passed through a fresh resin column at the same rate. The albumin and globulin fractions were prepared from oxalate plasma using ammonium sulfate and were dialyzed with Visking casing at 4°C until free of sulfate. *Prothrombin* was a citrate eluate of $BaSO_4$ adsorbate of oxalate plasma, dialyzed against citrate-saline for 18 hrs, 4°C, diluted with saline to 50 Iowa units/ml. *Thrombin* was prepared by a modified citrate activation procedure(4). *AHF* was prepared from f.e.-adsorbed plasma by a modified phosphate-citrate technic(5). The test for fibrinogen has been described(3). *Standard platelet suspension*: Silicone-treated glassware was used. Blood, drawn into an equal volume of EDTA solution previously diluted 1:10 with saline (0.9% NaCl), was centrifuged at 4°C 8-10 min, 425 *g*, and the platelet-rich supernate recentrifuged for 5 min, 2075 *g*. The supernatant plasma (EDTA plasma) was decanted. The platelet pellet was suspended by gentle agitation at 20°C in EDTA solution which had been diluted 1:100 with saline. After centrifugation, 2 resuspensions or "washings" were made in citrate-saline. The final suspension, after centrifugation to remove rbc's (7 min, 90 *g*), was counted with the phase microscope and diluted to the desired concentration, usually 4 x 10^5 platelets/cmm. *Citrate platelet suspension*: The standard procedure was modified by using citrate and citrate-saline instead of the 2 dilute EDTA solutions. *Resin platelet suspension*: The initial plasma-resin column (v.s.) was flushed with 50 ml saline. The platelet-rich wash was

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TABLE I. Effect of Platelet, Cation, and Plasma Variations on Platelet Agglutination.

Exp. No.	Platelet prep.	Plasma or derivative	Ion	Agglutination time (sec.)
		Platelets and ions		
1	Citrate	Resin	Mg	8
2	Resin	"	"	13
3	Standard	"	"	8
4	" , fresh	"	Mn	8
5	" , 28°, 4 hr	"	"	15
6	" , 28°, 20 "	"	"	19
7	" , 2°, 20 "	"	"	9
8	"	Oxalate	Mg	8
9	" , −20°, 1 hr	"	"	no aggl.
10	" , 37°, 5 min.	"	"	10
11	" , 50°, 5 "	"	"	27
12	" , 60°, 5 "	"	"	no aggl.
13	"	Resin*	Ca	"
		Plasma and plasma fractions		
14	Standard	Citrate	Mg	7
15	"	EDTA	"	16
16	"	Citrate†	"	8
17	"	$BaSO_4$-ads.†	"	8
18	"	Resin†	"	10
19	"	EDTA‡	"	13
20	"	Globulin	Mn	12
21	"	Albumin	"	no aggl.
22	"	Fibrinogen	"	8
23	"	Resin, 54°	"	40
24	"	Cit. f.e. ads.	Mg	7
25	"	$BaSO_4$-ads.	"	9
26	"	Prothrombin	"	no aggl.
27	"	Thrombin	NaCl	"
28	"	"	Mg	120
29	"	AHF	"	18
		Hemophilic plasma and platelets		
30	Standard	Hemophilic	Mg	10
31	"	Normal	"	10
32	" , hemophilic	"	"	10
33	" , "	Hemophilic	"	10

* Clotting time 4 min.

† Exp. 16, 17, 18: dialyzed *vs* cit.-saline for 4, 18, and 24 hr respectively.

‡ Dialyzed *vs* ox.-sal. for 24 hr.

Degree of agglutination was ++++ in all positive experiments except 6 (+++), 11 (++), and 28 (+).

centrifuged and the platelets twice resuspended in saline.

Procedure. Macroscopic platelet agglutination test was performed as follows: One ml plasma or plasma derivative was mixed with 0.25 ml cation solution (Ca, Mg, or Mn). After incubation for 30-40 min at 28°C, a 0.2 ml aliquot was aded to 0.2 ml platelet suspension and a stop watch started. The tube was tapped gently and the moment of appearance of platelet clumps was determined (agglutination time). Two minutes later the clumps were graded macroscopically on a scale of +, ++, +++, ++++. Microscopic examination of platelet aggregates revealed about 5 platelets per + aggregate, 10-25 per ++, 30-100 per +++, and over 100 per ++++ aggregate. Unagglutinated tests were observed at 2,10 and 30-60 min before being recorded as negative. The agglutination test was *modified* in some experiments by using 0.0108 M $MnCl_2$, in others by incubating 10 volumes of plasma or plasma dilution with 1 volume of 0.027 M $MgCl_2$ for 10 min. In these experiments the standard cation solutions were diluted with saline.

Results. I. Platelet Agglutination. Three components were essential for rapid agglutination—platelets, divalent cations, and plasma or plasma derivatives. Representa-

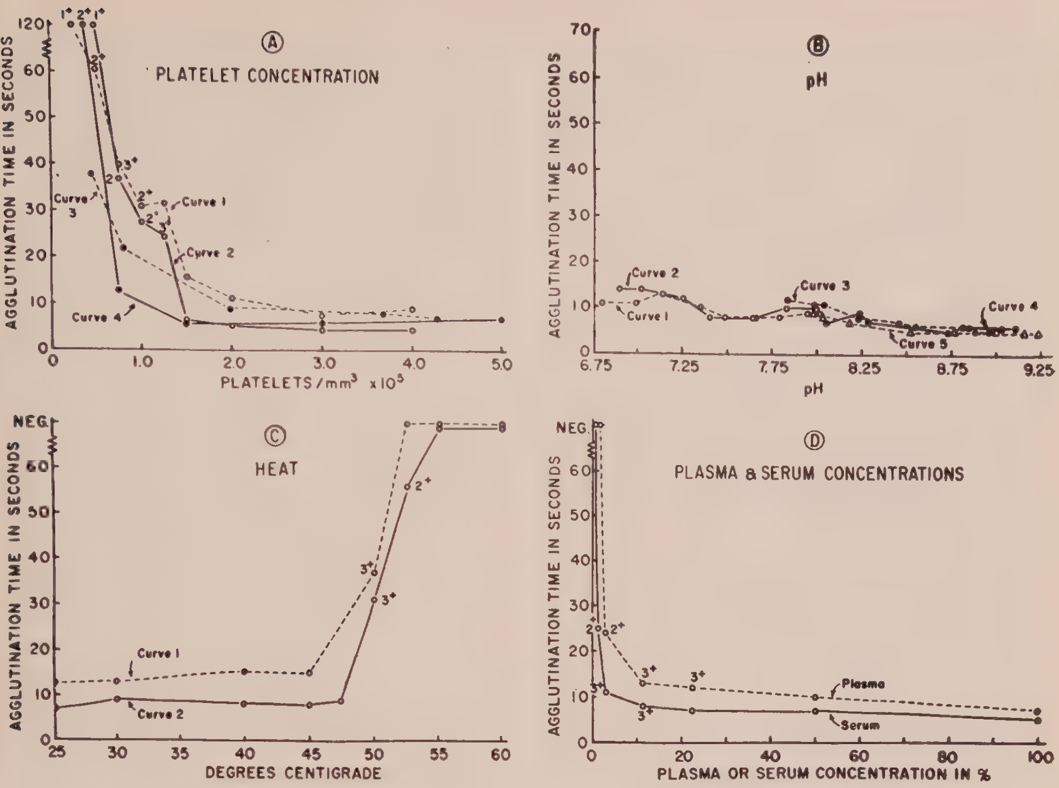

FIG. 1. Effect of platelet conc., pH, heating of plasma, and plasma conc. on platelet agglutination. Resin plasma was used throughout. Unless otherwise indicated, agglutination reactions were ++++. (A) *Platelet concentration.* Final conc. in agglutination test is indicated on abscissa. Curve 1: Test done with Mg. Curve 2: Test done with Mn. Curves 3 and 4: Modified agglutination test with Mg. (B) *pH.* pH was determined with a glass electrode immediately after agglutination. Modified test with Mg was used. Curve 1: 0.3 ml 0.2 M imidazole buffer of varying pH (3) was added to final agglutination tubes. Curve 2: Plasma was diluted with equal vol of imidazole prior to incubation. Curve 3: pH of plasma varied by adding 0.15 N NaOH prior to incubation. Curve 4: Plasma diluted with equal vol 0.2 M glycine buffer adjusted with N/5 NaOH; half-strength Mg. Curve 5: 0.2 ml glycine buffer was added to final agglutination tubes. (C) *Heat.* Plasma heated at indicated temperature for 10 min. Curve 1: With Mg. Curve 2: With Mn. (D) *Plasma and serum concentrations.* Dilutions were made with normal saline. Plasma or serum conc. on abscissa refers to their strength prior to use in test. Mg was used.

tive experiments in Table I and Fig. 1 illustrate the effect of variations in these 3 components. No agglutination was observed when either cation or plasma alone were tested.

Platelets. Various *anticoagulants* were used to prepare platelet suspensions. The results are shown in Exp. 1-3, Table I. In further experiments EDTA platelets gave the most reproducible results and became our standard. The platelets in these preparations were uniform biconvex discs when examined in the hanging drop with the phase microscope (970 x). *Washing* the platelets as many as 10 times did not alter their agglutinability. One preparation was divided into 6 lots, washed 1, 2, 3, 5, 7, and 10 times respectively, and tested with Mg and $BaSO_4$-treated plasma. Each lot reacted equally in the test. The influence of platelet *concentration* is shown in Fig. 1A. A final concentration of 2 x 10^5/cmm or higher gave best results, while agglutination times were longer and platelet clumps smaller with lower concentrations. *Storage* of platelet preparations at 28°C for 20 hr caused progressive reduction in their agglutinability (Exp. 4-6). If the suspensions were maintained at 2°C for as long as 20 hr and warmed to 28°C immediately before use, the platelets showed little change in reactivity (Exp. 7). Platelets frozen and thawed lost their agglutinability (cp. Exp. 8 and 9), as did those heated to 60°C (cp. Exp. 8 and 10-12).

Comparison of Ca, Mg, and Mn ions. In many experiments with plasma, either resin or $BaSO_4$-adsorbed, platelet agglutination was delayed or absent in the presence of Ca, but was rapid with Mg or Mn (cp. Exp. 13 and 3-4). The fact that Ca caused clotting, while Mg and Mn did not, suggests disassociation of platelet agglutination and coagulation.

pH. The effect of varying pH on platelet agglutination is shown in Fig. 1B. Between pH values of 6.8 to 9.25, agglutination times were in the narrow range of 5-14 sec. In our other studies, the pH was 7.6-7.9.

Plasma and serum. Prompt agglutination occurred with all plasmas tested, regardless of the anticoagulant used in plasma preparation (Exp. 3, 8, 14, 15). EDTA plasma gave the longest agglutination times. Plasma stored at –20°C retained its agglutinating activity. In one experiment, resin plasma which had been frozen for 3 months still had its original agglutinating power. Serum was found to be as active as plasma (Fig. 1D). The foregoing experiments suggest that a potent thrombocyte agglutinating factor(s) is present in plasma and serum.

II. Nature of Thrombocyte Agglutinating Factor (TAg) of Plasma. Additional experiments were undertaken to characterize this factor further. Fig. 1C shows that TAg was inactivated by heating at 52.5°-55°C. After dialysis the plasma factor was still present (Exp. 16-19). Plasma and serum retained high agglutinating capacities until diluted to concentrations below about 6-12% (Fig. 1D). On fractionation of plasma, TAg activity was found in the globulin but not in the albumin fraction (Exp. 20, 21).

In a final group of experiments, the possible identity of TAg with certain coagulation proteins was investigated. *Fibrinogen* preparations uniformly revealed high agglutinating activity (Exp. 22). On the other hand, serum, with no detectable fibrinogen, was equally active (Fig. 1D). Attempts were made to separate fibrinogen from TAg in plasma. Resin plasma heated to 54°C for 90 sec no longer clotted with thrombin, yet agglutination still occurred (Exp. 23). Citrate plasma from which fibrinogen had been removed by adsorption with fuller's earth(3) retained full agglutinating activity (Exp. 24). These experiments appear to separate TAg from fibrinogen.

The relation of TAg to *prothrombin* and *thrombin* was then studied. Agglutinating activity was present in $BaSO_4$-adsorbed plasma in which no prothrombin could be detected with either the 1-stage or the 2-stage tests (Exp. 25). Conversely, a prothrombin fraction of plasma had no TAg activity (Exp. 26). Thrombin which clotted fibrinogen in 2-3 sec caused no platelet agglutination (Exp. 27). However, the addition of cation caused variable agglutination (Exp. 28). Serum prepared by recalcification of resin plasma had no agglutinating activity (Exp. 13). This serum contained thrombin, as it clotted equal parts of fibrinogen in 49 sec. It thus appears that platelet agglutination by TAg is not dependent upon either prothrombin or thrombin.

An AHF fraction prepared from fibrinogen-free plasma showed moderate agglutinating activity (Exp. 29). On the other hand, canine hemophilic oxalate plasma, devoid of AHF, was as active as normal oxalate plasma (Exp. 30, 31). Thus TAg appears to be dissociated from AHF. In other studies suspensions of hemophilic platelets were agglutinated rapidly by both normal and hemophilic plasmas (Exp. 32, 33).

Discussion. Direct macroscopic observation of platelet agglutination provides a method by which rate and degree of agglutination can be readily determined. At the beginning of this study, agglutination times were in the range of 1-5 min. However, as optimum conditions for agglutination were determined, the times were shortened to 5-15 sec. Preparation of platelets was an important technical procedure in this study. The need for meticulous attention to such details as venepuncture and rapid mixing of the blood with anticoagulant cannot be overemphasized. Reports on use of EDTA for human platelet preparations suggest that altered forms regularly occur(6). In our standard preparations the platelets were discrete with regular outline and without spicules or similar alterations. The EDTA used was isosmotic with saline. The isolated platelets showed no inherent agglutinability, but were rapidly agglutinated by plasma-cation mixtures. This property of platelets could be easily destroyed by either freeze-thaw or heating procedures.

The finding that Ca is not essential for platelet agglutination was surprising in view of the many reports of its important role in this phenomenon [Lüscher(7)]. It was consistently observed that more rapid agglutination and larger platelet aggregates occurred with Mg or Mn than with Ca. Concentrations of Mg and Mn were isosmotic with saline. While rapid agglutination occurred under these conditions, the ions were greatly in excess of physiological concentrations. The pH for this reaction did not appear critical within the limits of 7-9.

In the study of the platelet agglutinating activity of plasma and serum, the same results were obtained with both autologous and homologous systems. The agglutinating factor appears to be a protein. It may be closely related to fibrinogen and AHF, judging from its presence in purified preparations of these clotting factors. However, other findings indicate that platelet agglutination is separate from the fibrin clotting process. In the systems where agglutination was rapid, as with Mn, no coagulation occurred. Further, systems free of fibrinogen, AHF, prothrombin and other $BaSO_4$-adsorbable coagulants were very active. Also, purified prothrombin had no TAg activity. Further study is needed to determine the basis of the agglutinating activity of the thrombin fractions. The presence of high agglutinating activity in canine hemophilic plasma confirms similar findings in human hemophilia(8). In view of the Roskam-Bounameaux hypothesis(9) that isolated platelets agglutinate because of procoagulants contained in plasma "adsorbed" to their surface, it was interesting that repeated platelet washings failed to alter their agglutinability. We have designated plasma activity as thrombocyte agglutinating factor(s) or TAg. Consideration was given first to the term viscous metamorphosis factor (VMF), but this was abandoned since VM has been so often related to the procoagulant plasma proteins. Also, this term has acquired many special connotations, including clot retraction(10), since its use by Eberth and Schimmelbusch(11).

Summary and conclusions. 1. Optimal conditions for platelet agglutination were determined in the dog by a macroscopic test. Most rapid agglutination was observed with Mg^{++} or Mn^{++}, undiluted plasma, and a platelet concentration above 200,000/cmm. Frozen or heated platelets were not agglutinable. 2. The thrombocyte agglutinating (TAg) factor found in plasma was thermolabile and nondialyzable. Under optimal conditions it caused gross platelet clumping in 5-15 seconds. This factor was active in the absence of fibrin coagulation, Ca^{++}, fibrinogen, AHF, prothrombin and related coagulant factors, suggesting the TAg may act independently of the coagulation process.

The authors are indebted to Dr. R. H. Wagner for the AHF fractions and to Dr. Barbara Moore for determinations of the freezing point of EDTA solutions.

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Comparative Activity of Isologous versus Homologous Mouse Bone Marrow on Lymphoid Tumor Production Following Irradiation.* (24051)

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Much recent work has been focused on protection of mice against lethal doses of irradiation by bone marrow and spleen(1-7). In an earlier paper(8), using thymic weight regeneration after sublethal doses of x-irradiation as an assay endpoint, only isologous or compatible[†] marrow was effective. Previous work on another effect of marrow injection, namely the inhibition of radiation-induced lymphoid tumors, utilized only isologous marrow(9,10). It seemed necessary to determine whether this marrow effect is also strain-specific. For this purpose, 2 inbred strains of mice and their F_1 hybrid were employed, since the genetics of tissue transplantation(11) predict that marrow from an inbred strain should be accepted (a) under isologous conditions, in other mice of the same strain, and (b) in a limited homologous situation in their hybrids; whereas hybrid marrow should be rejected by hosts of either parental strain and accepted only by isologous hybrids.

Materials and methods. Strain C57BL mice and reciprocal (C57BL x BALB) F_1 hybrids of both sexes were randomized into groups and exposed to fractionated total body irradiation starting at 33 ± 3 days of age. The C57BL mice received 4 doses of 168 r at 7-day intervals, and the F_1 hybrids 4 doses of 225 r at 7-day intervals.[‡] Immediately following last irradiation, 3 groups of each series were injected intravenously with a suspension of approximately 30-40 x 10^6 bone marrow cells (equivalent to contents of 2 femurs/0.5 ml Locke's solution/recipient) from C57BL, BALB, or F_1 hybrid donors, respectively, of the same sex and of approximately same age. An additional group in each series served as irradiated control, receiving no bone marrow. The animals were maintained under identical conditions, with free access to Purina laboratory chow and water. Any dead animal was routinely autopsied; representative viscera and lymphoid tissues were fixed for histological study, unless a typical lymphoma was grossly apparent. An attempt was made to watch particularly carefully those animals which had received homologous marrow. When labored breathing or enlargement of nodes or spleen indicated that a lymphoma was present, the animal was killed and autopsied; when possible, fragments of its tissue were transplanted subcutaneously into normal mice of the 3 genotypes, to determine whether repopulation of the irradiated host thymus by the injected bone marrow cells might have occurred.

Results. Results are presented in Fig. 1 and 2. The high incidence of lymphoid tumors arising in C57BL mice after this dose of irradiation alone (27 of 30 = 90%), was unaltered by injection of either BALB or F_1 hybrid homologous bone marrow (32 of 34, 34 of 36 = 94%), whereas isologous C57BL marrow exerted, as before, a powerful inhibitory influence (9 of 37 = 24%). By contrast, in F_1 hybrids, where 51 of 52 (98%) of irradiated controls developed lymphomas, both isologous hybrid marrow and compatible marrow from either parent were capable of inhibiting lymphoid tumor development (hybrid, 6 of 36 = 17%; C57BL, 4 of 29 = 14%, BALB, 11 of 38 = 29%).

Table I presents results of transplanting fragments of tumorous tissue, in most cases from the thymus, subcutaneously into normal animals of the 3 different genotypes. Transplantation of tumors arising in C57BL hosts

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[†] The term "isologous" as used here refers to marrow from animals of identical genotypes; "compatible" as used here refers to special case of parental marrow in a hybrid host, where, despite differences in genotype, the parental tissue can "take" without exciting an immune response from hybrid host.

[‡] Physical factors were 120 Kvp, 7.5 ma, 0.25 mm Cu + 1.0 mm. Al added filter, 30 cm target-mouse distance, output 24 r/minute.

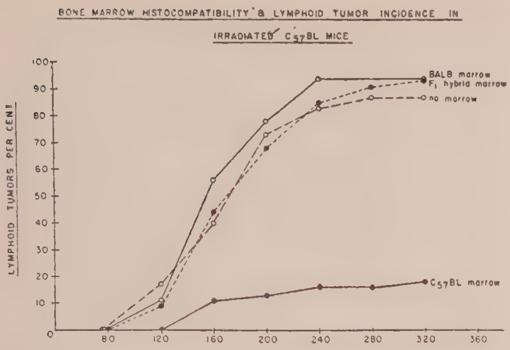

FIG. 1.

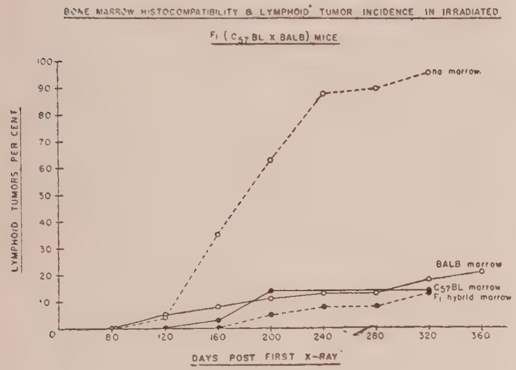

FIG. 2.

produced positive takes only in other C57BL mice, irrespective of marrow donor strain. However, similar tests of 9 tumors arising in F_1 hybrids after homologous parental marrow injection (1 C57BL, 8 BALB), led to positive takes in 3 instances in BALB mice, indicating that in these 3 cases repopulation of F_1 hybrid thymus by BALB marrow had occurred. Since it was not possible to test all such tumors, the frequency with which thymic repopulation by compatible homologous marrow may occur under these conditions is not established.

Discussion. It is clear that only isologous or compatible bone marrow is capable of inhibiting lymphoid tumor development at these radiation doses. This is, in fact, exactly the result obtained with respect to thymic weight regeneration after sublethal x-ray doses(8). The fact that sublethal radiation dose levels were employed is, however, an important limiting factor in interpretation of these data, since host immune mechanisms, though impaired after such doses, remain capable of destroying foreign cells(7). When this factor is taken into consideration, it becomes apparent that lymphoid tumor development was inhibited only in those donor-host combinations in which the injected marrow cells may be presumed to have remained viable.

It cannot properly be inferred from this, however, that direct repopulation of the thymus by injected marrow cells is necessarily the mechanism by which marrow facilitates thymic weight recovery or blocks lymphoma

TABLE I. Transplantation Behavior of Lymphoid Tumors Arising in Irradiated Marrow-Injected Mice.

Source of tumor	No. of tumors tested	Positive transplantation takes in C57BL	Hybrid	BALB	Remarks		
C57BL + C57BL marrow	0						
” + hybrid ”	14	28/35	16/35	0/14	1	tumor grew in	no host
					4	*Idem*	C57 only
					9	”	hybrids also
” + BALB ”	18	33/43	18/40	0/43	1	”	no host
					6	”	C57 only
					11	”	hybrids also
” (no marrow)	4	4/11	2/11	0/ 6	1	”	no host
					2	”	C57 only
					1	”	hybrids also
Hybrid + hybrid marrow	0						
” + C57BL ”	1	0/ 3	0/ 3				
” + BALB ”	8	0/13	17/26	7/28	5	”	hybrids only
					3	”	BALB also
” (no marrow)	6	0/15	3/16	0/16	4	”	no host

development. Indeed, the 3 cited instances of apparent hybrid thymus repopulation by parental marrow cells represent *failures* of protective marrow action, since tumors did in fact develop in those hosts. Parenthetically, it may be pointed out that the cells of these 3 tumors, which behaved on transplantation like parental cells, had never been exposed to irradiation; this confirms and extends our previously reported evidence for an indirect mechanism in the induction of these tumors (12).

Recently, Gengozian *et al.*(13) reported regular occurrence in mice exposed to lethal doses of radiation, of thymic repopulation by injected rat marrow cells, as demonstrated by immunologic responses, chromosome counts and morphology. It was of particular interest that thymic weight failed to return to normal levels in these animals, whereas mice protected with isologous marrow cells showed the usual prompt recovery of thymic weight. Merwin and Congdon(14) also noted atrophy in thymuses of irradiated mice despite immunologic evidence that homologous cells derived from injected marrow had lodged in those thymuses. Thus it would seem that homologous cells may repopulate the irradiated thymus but do not support its recovery to normal weight and histologic appearance. Since our thymic weight regeneration assay has to date consistently yielded results with respect to marrow specificity which parallel those for lymphoid tumor induction, it seems likely that foreign marrow will also fail to inhibit lymphoid tumor development at higher radiation dose levels. To settle this point, however, another experiment at LD_{99} radiation dose levels will be needed, comparing lymphoid tumor incidence in mice protected with isologous versus homologous marrow. Such experiments are made difficult by occurrence of late deaths in homologous marrow-bearing animals, leaving inadequate numbers available over the entire tumor induction latent period.

The histocompatibility of homologous parental marrow in F_1 hybrid recipients is not a reciprocal relationship. Uphoff(15) has called attention to a phenomenon which she terms "the F_1 hybrid effect": whereas isologous hybrid marrow confers excellent protection on F_1 hybrids exposed to lethal doses of irradiation, injection of parental marrow into such irradiated hybrids often results in late deaths. She attributes these to a delayed immune response of injected marrow cells against the host, as postulated by Billingham, *et al.* (16) and Barnes and Loutit(17). This response occurs only when the 2 parental strains have different histocompatibility genes. We, too, have observed this effect in a few radiation mortality experiments, using strains C57BL, BALB, and their F_1 hybrids, where virtually none of the C57BL marrow-injected hybrids survived beyond 75 days (unpublished) and a similar result with injected spleen cells has recently been reported by Schwartz, *et al.*(18). In the present experiment, where hybrids received a sublethal but greater than usual dose of irradiation, 11 of 47 mice injected with C57BL marrow died after the 30-day period generally associated with radiation deaths. Irradiated hybrids receiving BALB or hybrid marrow did not manifest such late deaths.

Summary. 1. Lymphoid tumor development in irradiated C57BL mice was inhibited specifically by isologous marrow; homologous marrow, including F_1 hybrid marrow, was incapable of inhibiting the tumor process in C57BL mice after sublethal radiation exposures. 2. F_1 (C57BL x BALB) hybrid mice were protected against radiation-induced lymphomas by compatible marrow. The protection was essentially equal, whether the marrow injected was from F_1 hybrid or from either parental strain. The similarity between these results and those previously reported with respect to marrow-mediated thymic weight regeneration is discussed. Since host immune reactions to incompatible cells are not abolished at these radiation dose levels, it is inferred that these effects of marrow injection require persistent presence of viable marrow cells. 3. A number of lymphomas were tested for transplantability into various strains. Of those tested, none arising in C57BL mice injected with homologous marrow were found to be other than C57BL tumors, whereas 3 of 9 tumors which arose in F_1 hybrids despite marrow injections, grew in the parental mar-

row donor strain, indicating that repopulation must have occurred in these cases at least.

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Effect on Serum Cholesterol in Man of Mono-Ene Fatty Acid (Oleic Acid) in the Diet.* (24052)

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From analysis of data from 41 sets of dietary comparisons in controlled experiments on groups of 12 to 27 men, we derived the equation: 1) Δ Chol. $= 2.74\ \Delta S - 1.31\ \Delta P$, for average change in serum cholesterol concentration when diet is changed in fats 2 to 4 weeks, ΔS being the difference between percentages of total calories provided in the 2 diets from glycerides of saturated fatty acids and ΔP being corresponding difference in glycerides of poly-unsaturated fats(1,2). This equation satisfactorily predicted group average responses of men in calorie equilibrium with constant amounts of protein and vitamins but with different amounts and kinds of fats in the diet. The original approach to this solution included provision for mono-enes (M) in the diet in the equation form: 2) Δ Chol. $= b\Delta S + c\Delta M + d\Delta P$. However, in all analyses the value of coefficient *c* proved not significantly different from zero, *i.e.* no influence of mono-ene could be proved, so final analysis was made in the form of Equation 1), above. After Equation 1) appeared, Ahrens *et al.*(3) published results from formula feeding experiments and more recently(4) they agree that their data satisfactorily conform to Equation 1). But they state that Equation 1), "could have been expressed just as accurately, and less mysteriously, in terms of iodine value of dietary fat" (ref. 4, p. 253). Further, they question the lack of effect of dietary mono-ene (oleic acid) on serum cholesterol level in man. *Iodine Value* vs. *2S-L.* Ahrens *et al.*(4) propose, in effect, that Equation 1) be simplified by approximation: 3) Δ Chol.$/1.35 = 2\Delta S - \Delta L$, rounding the coefficients and using L to refer to linoleic acid, since almost all the poly-unsaturated fatty acid in most ordinary food fats is actually linoleic acid. Further, they note that the iodine value of oleic acid (M) is 0.9/g, that of linoleic acid is 1.8/g, and the iodine value, $V = 0.9\,M' + 1.8L'$, where M' and L' are percentages of these fatty acids in total fat. It follows that V is a linear function of $S'-L'$ where $S' = 100-M'-L'$. But it does not follow that $S'-L'$ is substantially the same as $2S'-L'$ and an even more egregious error is the assumption that S' and L' are equivalent to S and P in Equation 2). S and P are percentages of total calories in the diet furnished by glycerides of saturated and linoleic (or poly-unsaturated) fatty acids; S' and L' are percentages of FAT made up by these acids. Assuming that the glycerides contain 95.6% fatty acids, and taking for illustration the situation where calorie equilibrium is maintained at 3000 Cal. daily, we can re-write Equation 3) for this situation: 4) $2.36\ \Delta\ \text{Chol.} = 2\Delta S''-\Delta L''$, where S'' and L'' are grams of saturated and linoleic acids. In the situation where 50 g each of saturated and linoleic acids in the diet are replaced by 100 g of oleic acid, there is no change in iodine value but from Equation 4) we have 4a) $2.36\ \Delta\ \text{Chol.} = 2(0-50) - (0-50) = -50$, and an average serum cholesterol fall of 21.2 mg/100 ml is predicted. In a 3000 Cal. diet as above, first 60 g each of saturated and linoleic acids and then only 10 g each of these could be fed replacing the 100 g of fatty acid with equivalent calories (941 Cal.) as carbohydrate. Again there is no change in iodine value, and hence no effect according to Ahrens *et al.*, but from Equation 4) we have: 4b) $2.36\ \Delta\ \text{Chol.} = 2(10-60) - (10-60) = -50$, and again the prediction is an average fall of 21.2 mg of cholesterol/100 ml of serum. Elsewhere(2) we indicated how iodine values *plus* values for total grams or calories of fat might be used to make predictions in cases like the above. But we also showed that an equation in these terms is theoretically and actually inferior because oleic acid is not simply half of linoleic acid in cholesterol effect.

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TABLE I. Composition of the Experimental Fats as g of the Glycerides/100 g of Each of the 3 Natural Fats and as g/32 g of Mixture of 52.8% Oleostearine and 47.2% of Safflower Oil.

(Analyses made by Research Division of Procter and Gamble Co., Miami Valley Labs.)

Class of fatty acid	Olive oil	Oleostearine	Safflower	Mixture
Saturated	13	67	12	13
Mono-ene	75	31	10	7
Poly-ene	12	2	78	12
Total	100	100	100	32

TABLE II. Composition of Fat Extracted from 4 Menus of Basic L.F. Diet as Served in Standard Portions to Provide 3200 Calories Daily.

	Menu 1	Menu 2	Menu 3	Menu 4	Mean
Iodine value	58.8	51.1	61.4	61.4	58.1
% of fatty acids					
Saturated	45.9	54.4	42.1	42.6	46.0
Mono-ene	44.6	36.4	49.5	47.9	44.6
Poly-ene	10.5	9.2	8.4	9.5	9.4

Methods. A critical test of the effect or lack of effect of oleic acid glyceride is to feed a diet in which it is isocalorically substituted for carbohydrate. Equation 1) predicts that there would be no significant change in serum cholesterol. One experimental fat diet (O.O.) was high in oleic acid provided by substituting, daily, 100 g olive oil for 900 Cal. of simple carbohydrates in the L.F. (low-fat) diet. The other experimental fat diet (S.O.) was made by substituting for 288 Cal. of carbohydrate in the L.F. diet, 32 g of a mixture of 47.2% safflower oil and 52.8% of oleostearine so as to match the O.O. diet in respect to saturated and poly-ene fats, but to be very much lower in oleic acid glyceride. Table I shows the composition of the several fats used. The calorie difference between O.O. and S.O. diets was balanced by carbohydrates. Gelatine was added to the O.O. diet to correspond with the very small amount of protein in carbohydrate foods in the S.O. diet but not in O.O. diet. Otherwise S.O. and O.O. diets were identical. 22 men aged 42 to 62 years were selected and controlled at Hastings State Hospital as previously described(2,5). After 2 weeks on L.F. diet they were divided into groups A and B, matched in regard to age, relative body weight, psychiatric diagnosis and habitual physical activity. Group A was transferred to the S.O. diet, Group B to the O.O. diet. After 2 weeks the diets of the groups were reversed, for a final 2 weeks.

TABLE III. Means of Actual Intakes on O.O. and S.O. Diets and Estimates for Low Fat Diet Based on Measured Servings on Low Fat Diet and Individual Adjustments as Made on O.O. and S.O. Diets. Values in g/day and as % of total calories. Also mean total daily calories for each period and mean body weights at ends of these periods.

	L.F. diet		O.O. diet		S.O. diet	
Item	g	% cal.	g	% cal.	g	% cal.
Protein	115.0	14.1	112.5	13.5	110.4	13.8
Fat	34.0	9.5	134.3	36.3	65.1	18.3
Saturated	15.2	4.2	30.6	8.3	29.7	8.4
Mono-ene	15.0	4.2	89.9	24.3	22.1	6.2
Poly-ene	3.8	1.1	13.8	3.7	13.4	3.7
Total calories	3260		3330		3190	

	Period I	Period II	Period III
Total calories per day	3260	3280	3240
Body wt (kg)			
Group A	67.8	67.5	67.8
" B	65.9	65.7	66.1

Blood samples were drawn from each man twice on 2 different days at end of each of 3 dietary periods. Serum total cholesterol was determined in duplicate, as described previously(6). In addition, serum phospholipid was estimated in duplicate on each sample. The serum sample (0.2 ml) was extracted with hot Bloor's mixture, filtered, the filtrate evaporated to dryness. The dry residue was extracted with hot chloroform and the analysis proceeded as in the method of Fiske and Subbarow(7). Table II shows the composition of the L.F. diet as served in 4 basic menus. In each case the complete menu was ground and homogenized, adding water as needed. Total lipid was measured in one set of aliquots, another being used for estimation of fatty acids. For the latter the slurry was lyophilized and the dry powder was saponified with alcoholic KOH, acidified and extracted at room temperature with ethyl ether. The ether was evaporated from the filtrate and the residue re-extracted with petroleum ether, the final analysis being made with official methods of the American Oil Chemists' Society. All of these manipulations were carried out at low temperature in an atmosphere of nitrogen. Table III shows average daily nutrients actually consumed on O.O. and S.O. diets and an estimate for nutrient intake on L.F. diet based on measured servings on L.F. diet and individual adjustments in Periods II and III. Master menus, from which basic servings were taken, were fixed at an average of 3200 Cal. in each of the 3 diets. The "adjustments" were small amounts of bread or jelly, or both, added to or removed from individual rations as needed to maintain weight constancy, and allowances for occasional plate waste. Small discrepancies between averages for O.O. and S.O. in Table II and the planned level of 3200 Cal. represent these adjustments which were recorded in Periods II and III but not in Period I (L.F.). In view of the high degree of weight constancy in all periods, it seemed safe to apply the average of these adjustments to Period I, but the effect is trivial in any case. It will be observed that amounts of saturated and poly-unsaturated fats eaten on O.O. and S.O. diets were substantially identical but the O.O. diet contained 67.8 g of oleic acid glycerides/day more than in the S.O. diet. Further, O.O. and S.O. diets provided about 3 times as much linoleic acid as the L.F. diet.

Results. Cholesterol and phospholipid data from this experiment are given in Table IV for each of 22 men at the end of each of 3 dietary periods. There was a general rise in serum cholesterol level when diet was changed from L.F. to either O.O. or S.O. Apparently this effect was incomplete at end of 2 weeks and progressed further during succeeding 2 weeks but, because of switchback design of the experiment, it is possible to compare the 2 experimental fats without interference by this trend. Mean cholesterol was 171.15 on S.O., 171.20 on O.O. diet. The mean for both groups on L.F. diet was 158.7 ± 4.9, while that on O.O. and S.O. diets was 171.2 ± 4.1. The difference between these means is 12.5 ± 6.3, and $p = 0.04$.

A more sensitive test of significance is to analyze results in terms of differences for each man on the several diets. The time trend from Period II to Period III complicates application of this method but correction can be made because it was not significantly different (+ 10.1 for Group A and + 10.0 for Group B). This method of individual differences also fails to show any difference in cho-

TABLE IV. Total Cholesterol and Phospholipid Concentrations, mg per 100 ml of Serum, at the Ends of the 3 Dietary Periods. Type of diet is indicated in parentheses at heads of columns.

Group	Cholesterol			Phospholipid		
	Nov. 12-14 (L.F.)	Nov. 26-27 (S.O.)	Dec. 10-12 (O.O.)	Nov. 12-14 (L.F.)	Nov. 26-27 (S.O.)	Dec. 10-12 (O.O.)
A	130	154	181	140	134	130
	166	170	168	107	124	125
	127	132	140	90	100	112
	182	180	190	120	145	126
	169	165	194	126	141	156
	136	142	150	106	110	120
	196	224	226	130	147	148
	187	166	199	130	114	131
	211	215	207	145	144	144
	156	140	143	111	110	102
	165	183	184	132	131	130
Mean	166.4	170.1	180.2	121.5	127.3	129.5
S.E.	± 8.0	± 8.9	± 2.2	± 5.0	± 5.0	± 4.7
	(L.F.)	(O.O.)	(S.O.)	(L.F.)	(O.O.)	(S.O.)
B	170	186	186	131	128	144
	148	163	185	128	136	152
	139	140	131	122	107	104
	152	140	174	115	110	140
	149	148	156	108	85	103
	133	150	148	101	114	114
	156	188	208	100	136	144
	144	174	170	118	124	132
	128	142	138	108	109	102
	152	180	186	99	122	132
	190	173	212	120	117	154
Mean	151.0	162.2	172.2	113.6	117.0	129.1
S.E.	± 5.2	± 5.7	± 8.1	± 3.4	± 4.4	± 6.0
A + B (N = 22)						
Mean	158.7	166.1	176.4	117.6	122.2	129.3
S.E.	± 4.9	± 5.2	± 4.2	± 3.1	± 3.5	± 3.7

lesterol values on O.O. and S.O. diets; the difference is zero and standard error is ± 3.1. Applying this method to comparison of L.F. with both O.O. and S.O. diets, shows that the mean: (O.O + S.O. diets) – (L.F. diet) = 12.5 ± 2.9, with $p < 0.001$.

The serum phospholipid values in general were parallel with the cholesterol values and there were no significant changes in cholesterol/phospholipid ratio, the averages being 1.4, 1.3, and 1.4 on the L.F., O.O., and S.O. diets, respectively. The mean phospholipid values were 117.6 ± 3.1, 123.3 ± 3.4 and 128.2 ± 3.8, on the L.F., O.O. and S.O. diets, respectively. The difference, S.O. – O.O., is 4.9 ± 5.1. The phospholipid rise in going from L.F. to the O.O. and S.O. diets is highly significant, the difference being 8.2 ± 3.0, with $p = 0.01$.

Discussion. The olive oil used is typical in fatty acid composition of the majority of olive oil samples considered by Hilditch(8) who notes, however, the report of Cattaneo *et al.* of a variation, according to maturity of the fruit, from 14.6% saturated and 6.6 linoleic, to 15% saturated and 22.5% linoleic. According to Equation 1), the former oil would have a small cholesterol-promoting effect when substituted for carbohydrate in the diet, whereas the latter would have very little effect. At a substitution level of 100 g/day and in balance at 3000 Calories, Equation 1) would predict an average rise in 2 to 4 weeks of around 10 mg of cholesterol/100 ml of serum from the former oil and only about 4 mg from the latter. It is understandable, therefore, that Bronte-Stewart *et al.*(9) could show no effect on serum cholesterol when olive oil was introduced into a low-fat diet for a few days in the diets of 2 Bantu men.

From a study of 2 persons on formula diets, Kinsell *et al.*(10) concluded that triolein and ethyl oleate are not equivalent in effect on cholesterol of half of the corresponding linoleic acid compounds. They cite us as arguing that "sufficient oleate would be quite as effective as linoleate." A major point in our refs. 1) and 2) was precisely the reverse. Malmros and Wigand(11) observed in 11 subjects a fall in serum cholesterol when olive oil was substituted for hydrogenated coconut oil in the diet and a further fall when corn oil was substituted for olive oil. These results are all predicted, at least semi-quantitatively, by Equation 1). Ahrens *et al.*(4) insist that effect of dietary fats on serum cholesterol is related to iodine value as closely as, or better than, our expression 2S-L, and they showed that for fats they used, there is a rough inverse correlation between iodine value and 2S-L *when total fat is constant.* The iodine values of fats in our diets were: L.F.—58, O.O.—75, S.O.—65 and the mean cholesterol values on these diets were L.F.—158.7, O.O.—171.2, S.O.—171.2. Obviously, in this set of diets the serum cholesterol levels bear no relationship to the iodine value.

Finally, it is interesting to apply Equation 1) in the prediction of cholesterol results in this experiment. First, of course, is the prediction of no difference between the O.O. and the S.O. diets, and this is confirmed. Second, Equation 1) predicts a rise in changing from L.F. to either O.O. or S.O. and this is also confirmed. The mean cholesterol rise from the L.F. diet to O.O. and S.O. diets predicted from Equation 1) is 7.4. The standard error of estimate of the prediction equation was S.E.E. = ±5.0 when tested with the 41 sets of data used in deriving Equation 1). Hence we may write: Δ Observed $= 12.5 \pm 2.9$, Δ Predicted $= 7.4 \pm 5.0$.

Summary. 1) A controlled dietary experiment was performed on 22 men who subsisted first on a low-fat diet and then, in a switchback arrangement of 2 matched groups of 11 men each, for periods of 2 weeks, on each of 2 diets differing by 67.8 g daily of mono-ene, the difference being matched by simple carbohydrate calories. The 2 diets were identical in all other respects and calorie equilibrium was maintained throughout all 3 dietary periods. 2) Results confirmed a prediction equation in showing no cholesterol difference on the 2 experimental fat diets, in spite of differences in iodine value and oleic acid content. Serum cholesterol rose significantly when the diet was changed from low fat to either of other diets and this change also conformed to that predicted by the equation. Serum phospholipid in general responded in parallel to cholesterol responses.

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Tissue Culture Studies of Auricular Appendages Obtained at Mitral Commissurotomy.* (24053)

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In some patients clinical and laboratory signs of rheumatic activity persist for many months despite the administration of penicillin to eliminate any hidden foci of streptococci and prophylactic medication to prevent reinfection. Such cases suggest the possibility that some agent other than group A streptococci may be a factor in prolonging activity of the rheumatic process. In recent years the finding of Aschoff bodies in a high percentage of the biopsies obtained at mitral commissurotomy from patients in whom there have been no signs of rheumatic activity for many years and in whom there is no evidence of a recent streptococcal infection, is pathologic evidence that the rheumatic process may remain active for decades(1,2). The persistence of this pathognomonic granuloma suggests the possibility of a viral agent.

In the past attempts to isolate a virus from patients with rheumatic fever have been unsuccessful. The discovery by Rowe and his coworkers(3) that a latent virus, (adenovirus) could be isolated from adenoid tissue obtained from routine adenoidectomies, suggested that other viruses might be demonstrable by similar technics. Tissue cultures of auricular appendages, therefore, were undertaken.

Material and methods. Specimens of auricular appendages were collected in the operating rooms of a number of cooperating hospitals.† The tissue was placed in Hanks balanced salt solution and taken to the laboratory where tissue cultures were prepared. At the same time a small piece of tissue was sent to the pathologic laboratory for preparation of histologic sections. Tissue cultures were also prepared from the foreskin of an 8-year-old boy and from cardiac tissue of 2 human fetuses. Specimens of auricular appendages, foreskin and fetal cardiac tissue were cut into fragments about one square mm in size and chicken embryo extract added. Four to 6 fragments were placed in each of 12 or more tubes which had been coated with chicken plasma. One ml of Eagle's basal medium(4) containing 100 units each of penicillin and streptomycin per ml, 25 units of mycostatin per ml and 10% calf serum by volume, was added to each tube. The tubes were incubated at 37°C without rotation. The medium was changed every 3rd or 4th day. Other specimens of auricular appendages were minced, ground in a mortar with alundum in a small amount of Hanks balanced salt solution. After centrifugation the supernate was added to actively growing fibroblasts derived from foreskin or fetal cardiac tissue.

Results. Specimens of auricular appendages were obtained from 17 patients varying in age from 26 to 57 years. Typical Aschoff bodies were found in 3 of these 17 specimens, (23%). The pathologic report of one specimen was: "rheumatic endocarditis, ? active, with focal myocarditis." No Aschoff bodies were found in the remaining 13 specimens and only hypertrophy of the cardiac muscle was noted in the pathologic reports.‡

Tissue Cultures of auricular appendages. Tissue cultures were prepared from 8 specimens. Cells resembling fibroblasts grew out after 6 to 8 days. These cultures were maintained for periods of 2 to 4 months, and were examined at frequent intervals. No cytopathogenic changes suggesting the presence of a viral agent were observed. None of the 8 specimens used in these experiments showed Aschoff bodies.

Extracts of emulsified auricular appendages.

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Supernates of emulsions of auricular appendages obtained from 2 patients were added to tissue cultures of actively growing fibroblasts derived from the foreskin of a normal 8-year-old boy. One of these auricular appendages showed Aschoff bodies, the other was negative. No inhibition of growth or cytopathogenic changes were observed in the cultures of the normal fibroblasts following addition of either of these supernates to the culture medium.

Supernates of emulsions of 2 auricular appendages, both of which showed Aschoff bodies, were added to actively grown fibroblasts derived from 2 human fetuses. Addition of this material to the culture medium did not impede growth or produce cytopathogenic changes.

Summary. Attempts to demonstrate a viral agent in auricular appendages obtained at commissurotomy from rheumatic patients with mitral stenosis were unsuccessful.

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Effect of Synthetic Lysine-Vasopressin on Adenohypophysial Activity in Toads. (24054)

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In various mammals administration of vasopressin releases adrenocorticotrophin from the pars distalis (anterior lobe) of the hypophysis(1-4). According to Guillemin and coworkers(5,6), the natural ACTH-releasing hypothalamic factor is a peptide that is perhaps chemically related to, but not identical with, the pars nervosa hormone vasopressin itself. Substances related to the mammalian pars nervosa hormones have been found in the neurohypophysis of all vertebrates(7-11).* Further, the function of the amphibian pars distalis is under hypothalamic control(12,13). This investigation was carried out to determine whether mammalian pars nervosa hormones are able to stimulate pars distalis activity in amphibians too.

Methods. The experiments were made on toads (*Bufo bufo* (*L*)) in which hypothalamic control of the pars distalis was prevented by interruption of the connections between hypothalamus and hypophysis. In such animals, as well as in hypophysectomized toads, moulting is inhibited, but not the keratinization of the epidermis. Eventually, therefore, the skin is thickly cornified. However, stimulation of the inactivated pars distalis causes shedding of all old cornification. Shedding of the hyperkeratinized skin was therefore used as a criterion of stimulation of the pars distalis. *Technic.* In 12 toads connections between hypothalamus and hypophysis were interrupted by extirpation of the median eminence, and the pars distalis was widely separated from the hypothalamic wound (Group A). The operation immediately interrupts the circulation in the pars distalis, but within a week or less blood flows again at a normal rate in the capillaries of the gland. The function of the gland, however, remains severely impaired, and moulting is inhibited when the

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* In amphibians and amniote vertebrates the neurohypophysis is differentiated into two separate structures, the pars nervosa (neural lobe) and the median eminence, whereas the neurohypophysis is undifferentiated in cyclostomes and most fishes(21,22).

TABLE I. Effect of Synthetic Lysine-Vasopressin and Insipidin AB on Moulting in Toads with Extirpated Median Eminence (Group A) or Median Eminence + Pars Distalis (Group B).

Group	U		No. of toads in which cornified layer of skin was Shed	Not shed
A	.1	lysine-vasopressin	12	0*
B	.1	lysine vasopressin	0	12
	1.	" "	0	6†
	.1	Insipidin AB	11	1‡

* In one of the toads hyperkeratinization of the epidermis did not occur, probably due to establishment of vascular connections between hypothalamus and pars distalis.

† Only half of the group was used in this exp.

‡ The toad was dying with symptoms of hypophysial deficiency(12).

blood supply is of extra-hypothalamic origin (12). Twelve toads with extirpated pars distalis and median eminence served as controls (Group B). All toads were females weighing 40-100 g. Synthetic lysine-vasopressin (7 U per ml) and the commercial vasopressin preparation Insipidin AB (20 U per ml) were injected subcutaneously.[†]

Results. 0.1 U lysine-vasopressin caused shedding of cornified layers in all toads of Group A, but in none of Group B (Table I). In Group B even 1 U lysine-vasopressin was without effect. Thus lysine-vasopressin induced moulting by stimulating the inactivated pars distalis. In Group B 0.1 U of commercial vasopressin Insipidin AB caused shedding of cornified layers in all toads but one. The commercial preparation, therefore, apparently contained enough pars distalis activity as an impurity to induce moulting directly in hypophysectomized toads.

Discussion. The present results provide further evidence for a relationship between pars nervosa hormones and pars distalis-controlling hypothalamic factors. In amphibians, birds, and mammals, hypothalamic control is mediated *via* the median eminence(12, 15-17). In dogs, ACTH-releasing activity has been demonstrated in blood drawn from the portal vessels that drain the blood from the median eminence into the pars distalis capillaries(18). However, the pars distalis controlling factors are not necessarily produced in the median eminence. In toads, for example, the factors most likely originate in hypothalamic centers, as do the pars nervosa hormones(20). Common chemical and functional properties of the hypothalamo—pars nervosa and hypothalamo—median eminence systems might have been anticipated because, phylogenetically, the pars nervosa and the median eminence are both derived from an undifferentiated neurohypophysis(21, 22).

The common phylogenetic origin of the two neurohypophysial structures of higher vertebrates raises the question about the sequence of origin of the various neurohypophysial functions. Two facts indicate that the specific functions of the pars nervosa are secondary and that the vertebrate neurohypophysis may primarily have been established as mediator of neural control of adenohypophysial function(24-26). These facts are 1) that all blood from the capillaries of the undifferentiated neurohypophysis of lower vertebrates is drained through the pars distalis, whereas the pars nervosa of higher vertebrates has a separate circulation; and 2) that numerous attempts to demonstrate typical pars nervosa (*e.g.* water balance) effects of neurohypophysial extracts in fish have given negative results(10,23). This theory is not incompatible with the demonstration of typical pars nervosa activities in the fish and even the cyclostome hypophysis when extracts of the gland have been tested on higher vertebrates (7,9,11). That extracts of the lamprey hypophysis, for example, show pressor, antidiuretic, and oxytocic effects in tests on rats may be due to chemical relationship between pars nervosa hormones of the rat and neurohypophysial pars distalis stimulating hormones of the lamprey. Thus functional evolution of the vertebrate neurohypophysis may have been accompanied by only small changes in chemical constitution of neurohypophysial hormones.

[†] We express our thanks to Dr. Vincent du Vigneaud, Cornell University Medical College, for supplying us with synthetic lysine-vasopressin, and to Dr. Aa. Theil Nielsen, Royal Danish School of Pharmacy, for checking the pressor activity of the preparation.

Summary. In toads (*Bufo bufo* (*L.*)) with deficient pars distalis function due to extirpation of median eminence, subcutaneous injection of synthetic lysine-vasopressin causes shedding of the hyperkeratinized stratum corneum. In hypophysectomized toads lysine-vasopressin is without effect. Lysine-vasopressin is thus able to stimulate pars distalis activity in toads, indicating a chemical relationship between pars nervosa and median eminence (adenohypophysiotropic) hormones.

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Histopathology of Lymphocytic Choriomeningitis in Mice Spared by Amethopterin. (24055)

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Amethopterin, a folic acid antagonist, spares the lives of mice infected with normally fatal lymphocytic choriomeningitis (LCM), but does not prevent virus replication in spared mice. Folic acid-deficient diet produces a similar effect(1,2). These alterations resemble those obtained earlier with whole body X-irradiation; in mice spared by this treatment, decreased tissue reactivity or diminished inflammatory cellular exudate were suggested as the explanation for survival (3). The present report indicates that mice spared by amethopterin tend to develop inflammatory reaction more slowly than untreated mice, most of them showing great modification of the natural disease process, but that a significant number survive even though extensive inflammatory reaction is present.

Materials and methods. The LCM virus used was a murine strain maintained through more than 400 serial intracerebral (i.c.) passages(4). Preparations were mouse brain emulsions (ground by hand in a mortar and uncentrifuged) made to 10% suspensions and decimally diluted in 0.85% buffered saline. The i.c. dose was 0.03 ml for all animals. Mice were N.I.H. Swiss "general purpose," of both sexes, weighing 16-18 g when inoculated. They were fed a stock diet consisting of whole

TABLE I. Incidence of Lesions and Virus Recovery in Untreated Mice Given LCM Virus Intracerebrally.

	No. mice	Log dilution virus given	Days post inoc.	Lesions in:			Log highest dilution virus recovered*
				Central nervous system	Lymph nodes & spleen	Viscera	
	1	–5	5	+	+	—	–5
	4	–5	6	+	+	+	–7
		–4	6	+	+	+	–6
		–5	6	+	±	—	–7
		”	6	+	+	—	–7
	3	–4	7	+	+	—	–5
		–5	7	+	+	—	–7
		”	7	+	+	±	–6
Total	8			8 (100%)	7	2	

+, typical of acute LCM. ±, modified or equivocal. —, minimal or negative.

* Highest dilution which killed 3 or more out of 5 mice inj. intracer. Titrations as performed did not establish end-points.

wheat bread (fortified with thiamine, riboflavin, niacin, and iron) soaked in powdered whole milk reconstituted with tap water. Amethopterin, 0.06 mg in 0.2 ml of 2% $NaHCO_3$, was given intraperitoneally on days 3, 5, 7, and 9; virus having been given on day 0. No mouse received amethopterin on the day it was killed; *e.g.,* an animal killed on day 7 received amethopterin on days 3 and 5 only. All studies were on killed mice. The brain of each was divided sagitally *in situ,* and half removed for virus titration, the remainder being left in the cranium for fixation, along with the body of the mouse, in 10% buffered formalin. (The cerebral hemisphere which had been injected with virus was the one left for study.) Brain, meninges, choroid plexus, spinal cord, all viscera, lymph nodes, and bone marrow were stained with buffered azure-eosin and examined histologically. Virus titrations employed 5 mice for each log dilution.

Results. 1. *Untreated Mice.* Eight untreated mice killed from 5 to 7 days after i.c. injection of virus (2 with 10^{-4} dilution; 6 with 10^{-5}) showed typical lesions of severe, acute lymphocytic choriomeningitis in the central nervous system. Seven of these mice had changes in lymph nodes and spleen, and 2 in viscera. In 7 mice, virus titrations failed to reach end-points at 10^{-6}; in one, titration was carried only to 10^{-5}, which killed all mice. Results are summarized in Table I. No untreated mice survived beyond 7 days. Lesions in mice after intracerebral inoculation of LCM virus have been described by Lillie and Armstrong(5,6), and the changes observed are noted here only briefly, for purposes of comparison with those in treated animals. Infiltration by large numbers of lymphocytes accompanied by a few macrophages and very few polymorphonuclear leukocytes was intense in the meninges over the superior surface and base of the brain and frequently in the sheaths of penetrating submeningeal blood-vessels, and to a lesser degree in the spinal meninges. The choroid plexus in all locations contained varying amounts of lymphocytic exudate, as did the ependymal lining of the ventricles. Lesions in lymph nodes, spleen, and viscera were less consistent in frequency and severity. Involved lymph nodes, most frequently those of the cervical group, showed hyperplastic follicles, numerous phagocytic cells, considerable reticulo-endothelial hyperplasia which occasionally was so extensive as to obliterate follicular architecture, and occasionally perinodal infiltration by lymphocytes. The spleen when involved showed changes similar to those in lymph nodes, as well as occasional congestion of the pulp, or hemorrhage with necrosis and destruction of follicles. Other viscera much less frequently showed focal and interstitial lymphocytic infiltration; of these, the liver and kidney were most often involved. This was in accord with the previous observations of considerable variability in tissues and

TABLE II. Incidence of Lesions and Virus Recovery in Amethopterin-Treated Mice Given LCM Virus Intracerebrally. Acute stage.

				Lesions in:			
No. mice	Log dilution virus given	No. doses drug given	Days post inoc.	Central nervous system	Lymph nodes & spleen	Viscera	Log highest dilution virus recovered*
1	–5	1	5	—	—	—	–5
4	–5	2	6	—	—	—	–6.5
	–4	2	6	—	—	—	–6
	–5	2	6	—	±	—	–5
	–5	2	6	—	—	—	–6
4	–5	2	7	—	+	—	–6.5
	–4	2	7	—	—	—	–7
	–5	2	7	—	+	±	–7
	–5	2	7	—	±	±	–7
3	–4	3	8	—	±	±	–6
	–5	3	8	+	+	—	–7
	–5	3	8	+	+	—	–7
2	–5	3	9	±	±	—	–7
	–5	3	9	—	+	—	–6
3	–4	4	10	+	±	—	–7
	–5	4	10	—	—	—	–7
	–5	4	10	—	±	±	–7
2	–5	4	13	—	—	±	–7
	–5	4	13	+	—	±	–7
1	–4	4	14	—	+	±	–7
1	–5	4	17	—	+	±	–6
1	–4	4	20	—	—	+	–7
Total 22				4 (18%)	7	1	

+, typical of acute LCM. ±, modified or equivocal. —, minimal or negative.

* Highest dilution which killed 3 or more out of 5 mice inj. intracer. Titrations as performed did not establish end-points.

organs involved from mouse to mouse.

2. *Treated mice*: *acute stage*. Twenty-two mice were sacrificed from 5 to 20 days after LCM virus injection (6 given 10^{-4} dilution; 16 given 10^{-5}). Only 4 mice showed intense lymphocytic infiltration of the meninges and choroid similar to that seen in the untreated control animals, and were considered to have severe active lymphocytic choriomeningitis. The changes in lymph nodes, spleen, and viscera in these 4 mice were less consistent and less frequent than those observed in the control animals (Table II). The remaining 18 animals showed extraordinary modification of the natural disease. Of this group, some were completely devoid of histological evidence of lymphocytic choriomeningitis. Others showed changes varying from extremely slight traces of LCM to greatly modified disease suggesting healed or healing LCM. These changes were manifested by minimal infiltrations by a very few lymphocytes to slight amounts of lymphocytic infiltration of meninges or of choroid, and similar slight infiltrates in the linings of the lateral, 3rd, or 4th ventricles. Lesions in the lymph nodes, spleen, and viscera were infrequent, generally less severe than in untreated animals, and were not consistently associated with the presence of modified central nervous system lesions when the latter occurred.

Since the central nervous system was the only consistent site of characteristic pathological changes in control animals, and since the virus had been introduced by the intracerebral route, the presence or absence of lesions in this location was considered the most reliable index of active, modified, or absent cellular response.

In 20 of these mice, virus titrations at 10^{-6} failed to reach end-points; in the other 2, tests carried out to 10^{-5} killed all mice. This

TABLE III. Incidence of Lesions and Virus Recovery in Amethopterin-Treated Mice Given LCM Virus Intracerebrally. Chronic stage.

					Lesions in:			
	No. mice	Log dilution virus given	No. doses drug given	Days post inoc.	Central nervous system	Lymph nodes & spleen	Viscera	Log highest dilution virus recovered*
	2	−5	4	21	+	+	—	−7
		−5	4	21	+	—	—	−6
	1	−4	4	26	+	+	+	−5
	2	−5	4	27	+	±	±	−7
		−5	4	27	+	+	—	−5
	1	−5	4	28	±	—	+	−5
	1	−4	4	29	+	+	+	−5
	1	−4	4	32	—	+	±	−5
	1	−4	4	42	±	±	±	<−3†
	1	−4	4	43	+	+	±	−7
	1	−4	4	56	+	+	+	−7
	1	−5	4	57	—	+	—	<−3†
Total	12				8 (67%)	8	3	

+, typical of acute LCM. ±, modified or equivocal. —, minimal or negative.

* Highest dilution which killed 3 or more out of 5 mice inj. intracer. Titrations as performed did not establish end-points.

† Brains from mice killed on days 42 and 57 killed 1 out of 5 and 2 out of 5 respectively at 10^{-3} dilution.

is taken to indicate virus replication in all the animals studied in this group. Results are shown in Table II.

3. *Treated mice: chronic stage.* Twelve mice were sacrificed from 21 to 57 days after LCM virus injection (6 given 10^{-4} dilution; 6 given 10^{-5}). Of these, 8 showed the characteristic heavy lymphocytic infiltration of meninges and choroid similar to that seen in control animals. Changes in lymph nodes, spleen, and viscera in these 12 mice were similar in severity and frequency to those seen in the untreated controls. (Table III).

Four mice showed modification of the natural disease, varying from extremely slight traces of lymphocytic choriomeningitis to greatly modified disease, similar to the 18 mice described in Section 2. Despite the length of time after inoculation, those animals showing evidence of active disease had extensive histological lesions even as late as 56 days post injection, similar and comparable in some cases to the extent and severity of the lesions observed in untreated control animals at the acute stage.

Virus recoveries in titration tests, in dilutions of 10^{-5} to 10^{-7}, without reaching end-points, indicated virus replication in these mice. Results are given in Table III. The two instances where virus recovery is shown as less than 10^{-3} (42 and 57 days post infection) do not prove virus replication, but do not exclude it.

Discussion. The characteristic pathological picture of lymphocytic choriomeningitis was completely eradicated or markedly suppressed in mice given amethopterin after intracerebral inoculation with normally lethal doses of virus, in 82% of animals in the acute stage, and in 33% in the chronic stage. The difference in incidence of lesions of active LCM at these 2 stages tends to indicate that this treatment actually may have delayed rather than suppressed the inflammatory reaction, to a large degree. If the reaction which occurred in 67% of mice in the chronic stage was delayed by amethopterin, it is possible that the early effect of amethopterin had abated, but that some degree of immunity had been acquired from exposure to the virus. This alteration of response may be presumed to occur with folic acid-deficient diet, and has been shown to follow whole body X-irradiation, both of which have similar life-sparing effects. In the studies reported here, amelioration of the pathological process was not essential for survival, since a significant number of spared mice showed an active cellular response. The sparing effect was not attributable to eradication or diminution of virus, since spared mice with and without active lesions showed evidence of virus multiplication (Tables I, II, III).

The host-parasite relationship becomes somewhat obscured by these findings, since it has been generally assumed that an inflammatory cellular response to an invading microorganism is beneficial to the host, by providing under some circumstances a defensive mechanism for the arrest, destruction, or elimination of the invader. Rowe has suggested that in lymphocytic choriomeningitis the cellular response may play an opposite role, being responsible for mortality, and that its suppression therefore allows survival(3). In the present study, resistance to normally lethal doses of virus occurred whether or not a cellular response developed, and in the presence of consistently active virus multiplication in the host.

Since amethopterin is one of a class of compounds which are capable of interfering with various aspects of metabolism and growth, alteration of the metabolic activity of the virus as well as its synthesis or destruction must be considered as possible determining factors in the outcome of LCM infection in treated mice. It is conceivable that modification of viral activity might in some fashion affect its lethal capacity, but if such a factor were operative it could be effective only while the virus was in the amethopterin-treated mouse. However, it seems likely that the virus itself was not affected, since its multiplication in the host was not impeded and its infectivity and lethality for new mice appeared unimpaired, in spite of the alteration of the pathological picture and suppression of the lethal effect.

Another possibility is that interference with the cellular metabolism or cellular production of the mouse could alter the outcome of the infection. The influence of humoral responses of the host is suggested by the observations that spared mice are refractory to subsequent challenge with the virus(1,2). In order to determine the significance of modification of the cellular response on the course of this infection, it would be necessary to prevent cellular response by procedures which could impede cellular production or the exudation of pre-existing cells, and yet not interfere with viral metabolism or growth, or with other responses (conceivably humoral) of the host.

Summary. Mice infected with lymphocytic choriomeningitis virus in doses which were otherwise uniformly fatal were spared by administration of amethopterin. Histological examination of spared animals at intervals ranging from 5 to 57 days revealed greatly modified objective evidence of disease in the majority of these animals. In the acute stage, 82% of the mice showed no disease or extremely modified lesions of LCM. In the chronic stage, 33% showed similarly modified LCM. The remaining mice in both groups showed lesions of active LCM, even as late as 56 days after inoculation. Practically all spared mice gave evidence of multiplication of the virus in the central nervous system.

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Distribution of 3-Methylglucose in Organs of Rat.* (24056)

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We reported that in the rat 3-methylglucose is eliminated unchanged almost quantitatively in urine within 24 hours. However, even after this time traces of 3-methylglucose could be found and identified by paper chromatography in brain and muscle but not in plasma and liver(1). This apparent retention by some tissues and the fact that 3-methylglucose is a non-metabolized compound(2) prompted a detailed study of the distribution of this substance in tissues of the rat.

Materials and methods. Sprague-Dawley male rats were kept on standard laboratory diet and starved 24 hours prior to experiments. 3-methylglucose was the same preparation used in previous study(1). The Nelson copper reduction method was used to determine 3-methylglucose(3). Glucose was previously removed from plasma by treatment with washed suspension of Baker's yeast(4). *Preparation of tissue extracts for quantitative studies.* The excised tissue was weighed and homogenized in methanol and the resulting suspension was adjusted with methanol to known volume and filtered. An aliquot of filtrate was evaporated to dryness in vacuum at 60°C. The residue was dissolved in known small amount of water and subjected to chemical assay. Water content of organs was determined by drying to constant weight in electric oven at 105°C.

Results. Distribution of 3-methylglucose in tissues of nephrectomized rats. Both kidneys were removed in ether anesthesia. The 3-methylglucose solution was injected into exposed femoral vein. Following injection, the blood level of this sugar became constant in about 2 hours. At this time animals were bled from abdominal aorta into syringes containing heparin. 3-methylglucose was assayed in plasma and in the following organs: brain, liver, gastrointestinal tract, cardiac and voluntary muscles. Values obtained have been corrected for non 3-methylglucose reducing substances which are present in tissue extracts of normal animals.

Table I presents the calculation of "apparent volume of distribution" or "3-methylglucose space" based on plasma analyses. This value is 35-37% of body weight. More detailed information can be obtained if distribution of 3-methylglucose in various organs is calculated as is shown in Fig. 1. The values on this chart are relative: the 3-methylglucose content of plasma water is expressed arbitrarily as 100. Liver and myocardium water contain about the same concentration of 3-methylglucose as plasma-water, whereas brain, gastrointestinal tract and leg-muscle water contain only about ⅓ to ¼ as much.

TABLE I. Apparent Volume of Distribution (r) of 3-Methyl Glucose in 3 Nephrectomized Rats.

Wt, g	3-methyl glucose, mg	Plasma conc., mg %	r, % body wt
190	380	536	37.3
218	440	573	35.2
208	420	556	36.3
		Avg	36.3

Elimination of 3-methylglucose from organs of rat. Four g/kg 3-methylglucose were injected intravenously into intact animals. They

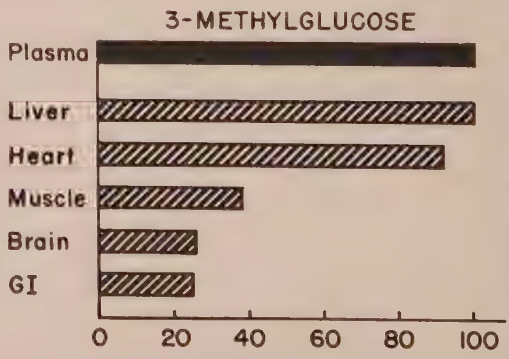

FIG. 1. Relative distribution of 3-methyl glucose in the water of various organs from nephrectomized rats. Concentration in plasma is expressed arbitrarily as 100. GI = gastrointestinal tract.

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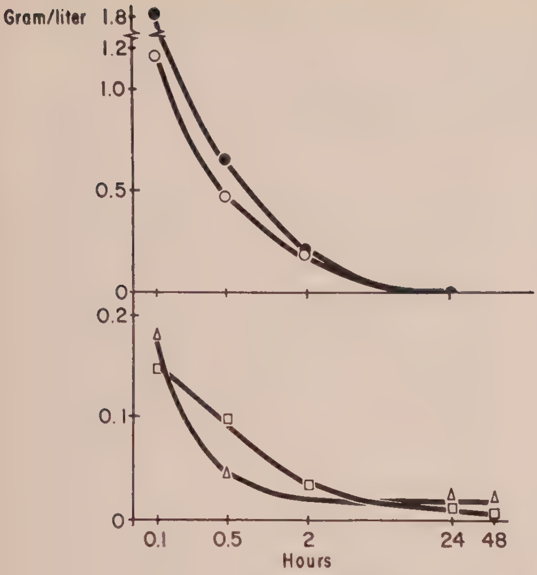

FIG. 2. 3-methyl glucose concentrations in tissue water of rats following intrav. injections. Ordinate: sugar concentrations; abscissa: time following inj. ●——● kidney, ○——○ liver, □——□ brain, △——△ leg muscle. Note difference in scale on ordinate of the upper and lower curves.

were then killed at intervals of 5 minutes, 30 minutes, 2 hours, 24 hours, and 48 hours. The organs were extracted and assayed for 3-methylglucose as described above. Fig. 2 illustrates results obtained in 4 organs including kidneys. Liver and kidney are shown on same coordinate as the concentration of 3-methylglucose, as their tissue water was rather high initially and then fell rapidly to reach 0 at 24 hours. With voluntary muscle and brain, the original concentration was low but elimination was slower.

Discussion. The apparent volume of distribution of 3-methylglucose in rats is about 35-37% of body weight. For glucose a value of 30-31% was calculated in intact rabbits(5). Distribution is uneven; some organs, like liver and cardiac muscle, reach equilibrium with plasma, whereas, in other tissues, the rate of passage of 3-methylglucose is slow in both directions. The latter organs thus still contain small amounts of 3-methylglucose 24 or even 48 hours following single intravenous injection. The difference in glucose uptake between cardiac and voluntary muscle was pointed out by Cori *et al.*(6) and was thought to be due to a larger number of open capillaries in heart muscle than in leg muscle. However, the finding that 1-methyl fructose penetrates more readily into leg muscle than into the myocardium points rather to specific functions of the membranes of corresponding muscles.

The 3-methylglucose space in muscle is larger than, for example, the chloride space. Moreover, slow release of this sugar by muscle tissue indicates that a part of 3-methylglucose is in the intracellular water of the muscle.

Summary. The apparent volume of distribution of 3-methylglucose in rats is 35-37% of body weight. Liver and myocardium water reaches equilibrium with plasma water; other organs are less permeable to this sugar.

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Liver Glycogen in Potassium-Deficient Rats Following Carbohydrate and Alanine Administration, With and Without Potassium. (24057)

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Numerous reports involving both *in vivo* and *in vitro* studies have indicated a relation between carbohydrate and potassium metabolism(1,2). It has been appreciated for many years that glycogen deposited in liver is accompanied by water, and Fenn(3) demonstrated that there is also simultaneous deposition of potassium. Studies with potassium-deficient rats have revealed elevated fasting liver glycogen, normal fasting blood sugar and normal oral glucose tolerance curve (4,5). Intraperitoneal injection of potassium with glucose to normal rats resulted in lesser liver glycogen deposition than when glucose without potassium was injected(6). The present experiments were therefore undertaken to study further the influence of availability of potassium on extent of liver glycogen deposition. For this purpose fasting control and potassium-deficient rats were given by stomach tube glucose, fructose and alpha-alanine, with and without potassium, and liver glycogen determined 4 hours thereafter.

Methods. Young adult male rats of Wistar strain, of uniform age and weighing between 250 and 298 g, were placed on control and low-potassium diets (as in previous studies(7,8)) for 5 weeks. After withdrawing food 48 hours and allowing water *ad libitum*, members of both groups were sacrificed by removing the heart following sodium pentobarbital anesthesia (50 mg/kg body wt., subcutaneous). The liver was quickly excised, blotted free of adhering blood, weighed and sampled at once for chemical analysis. Other members of both groups of rats were simultaneously given, by stomach tube, either 5 ml of 40% glucose or 5 ml of 40% glucose containing 1.2 meq potassium. The animals were then sacrificed 4 hours later. The experiments involving administration of fructose and alanine, without and with potassium, were carried out in same manner. For fructose administration 5 ml of 40% fructose and 5 ml of 40% fructose containing 1.2 meq K were given, while for alpha-alanine administration 5 ml of 20% alpha-alanine and 5 ml of 20% alpha-alanine containing 1.2 meq potassium were given. Liver glycogen was determined by the anthrone reagent(9). All other chemical analyses were carried out in essentially the same manner as previously reported(7,8).

Results. Liver glycogen. In agreement with the findings of others(4,5) the liver glycogen content of fasting potassium-deficient rats was appreciably greater than that of fasting control rats (Table I). There was an unimpaired deposition of liver glycogen in potassium-deficient rats given either glucose, fructose or alpha-alanine. Further, simultaneous administration of potassium appeared to have no significant effect on corresponding levels of liver glycogen.

Liver water and potassium. Liver water/kg fat-free tissue remained relatively constant. As pointed out in earlier studies(3,10,11) a constant percentage of liver water with glycogen deposition indicates addition of water. Likewise, an absence of fall in liver potassium with glycogen deposition indicates addition of potassium(3). A comparison of changes in liver glycogen, water and potassium from corresponding control levels can be made, if it is assumed that during the 4 hours experimental period there was no significant change in total N. On the basis of constant N content, changes in liver water, glycogen and potassium were similar in the 2 groups of animals. Further, it made no difference whether or not potassium was simultaneously administered. In rats fed low-potassium diet with apparent marked skeletal muscle potassium deficiency(7) potassium was made available from some source, so that the ratios of changes in liver potassium to glycogen were in ranges found in normal animals. Although the values were approximately the same within corresponding groups of animals, the increment of liver water/g glycogen showed some

TABLE I. Liver Glycogen of Normal and Potassium-Deficient Rats.

Exp.*	No. of rats	Body wt, g	Liver wt, g	Liver fat, %	Liver/kg fat-free tissue					Content/g liver N			Change/g liver N			ΔH_2O	ΔH_2O	ΔK
					Water, g	Glycogen, g	K, meq	Na, meq	N, g	Water, g	Glycogen, g	K, meq	Water, g	Glycogen, g	K, meq	Δ Glycogen	ΔK	Δ Glycogen
C	22	323 31.6†	7.22 .674	3.67 1.049	726.5 3.67	5.7 4.02	104.7 3.76	32.6 2.27	37.7 .90	19.27	.151	2.777						
C + G	5	314 19.8	7.71 .776	2.68 1.027	724.9 5.76	38.8 18.20	106.4 1.97	30.4 1.48	33.6 1.08	21.57	1.155	3.167	2.30	1.004	.390	2.29	5.90	.39
C + G + K	6	326 24.3	7.67 .722	3.46 .563	726.4 3.37	33.3 5.71	106.2 2.76	29.5 1.92	34.8 1.66	20.87	.954	3.052	1.60	.803	.275	1.99	5.82	.34
C + F	6	329 23.4	8.36 .882	3.12 .580	725.4 2.48	43.4 7.94	108.2 2.18	31.4 1.39	34.5 1.37	21.03	1.258	3.136	1.76	1.107	.359	1.59	4.90	.32
C + F + K	6	347 30.6	8.95 .815	3.38 .679	729.4 6.78	44.9 12.31	107.5 4.12	32.0 2.86	33.5 .77	21.77	1.340	3.209	2.50	1.189	.432	2.10	5.79	.36
C + Ala	6	333 27.7	7.98 .598	3.84 .586	733.8 5.74	19.6 8.00	104.2 3.49	32.1 1.41	36.3 .71	20.21	.540	2.871	.94	.389	.094	2.42	10.00	.24
C + Ala + K	6	348 14.0	8.13 .380	4.03 .797	730.7 6.66	14.7 6.03	105.6 3.35	32.6 1.02	37.0 1.18	19.75	.397	2.854	.48	.246	.077	1.95	6.23	.31
LK	22	250 12.8	6.76 .584	2.04 .863	727.2 5.35	13.8 6.09	102.0 3.11	39.0 2.02	36.7 1.14	19.81	.376	2.779						
LK + G	5	253 20.2	7.20 .784	1.23 .231	724.8 4.86	42.0 16.31	106.5 1.25	34.8 1.42	33.4 1.55	21.70	1.257	3.189	1.89	.881	.410	2.15	4.61	.47
LK + G + K	6	253 20.8	7.32 .990	1.42 .076	722.2 4.17	44.1 9.68	110.8 3.88	33.0 1.06	33.0 2.60	21.88	1.336	3.358	2.07	.960	.579	2.16	3.58	.60
LK + F	5	234 27.2	7.33 1.368	1.63 .307	719.9 5.34	59.8 20.29	106.9 8.03	35.4 .92	34.0 3.03	21.17	1.759	3.144	1.36	1.383	.365	.98	3.73	.26
LK + F + K	6	247 17.9	7.96 1.000	2.17 .339	720.8 5.49	54.2 10.96	110.7 2.09	33.9 1.21	33.5 2.54	21.52	1.618	3.304	1.71	1.242	.525	1.38	3.26	.42
LK + Ala	5	226 19.2	6.35 .671	2.47 .249	728.5 5.19	22.8 9.28	106.2 4.23	36.6 1.71	36.5 1.09	19.96	.625	2.910	.15	.249	.131	.60	1.15	.53
LK + Ala + K	6	251 12.7	6.77 .673	2.62 .345	729.9 4.97	21.3 4.64	106.3 2.36	36.9 1.12	36.6 1.37	19.94	.582	2.904	.13	.206	.125	.63	1.04	.61

* Control (C) or low-potassium (LK) diets for 5 wk, and fasted 48 hr. Glucose, 2 g (G); fructose, 2 g (F), or alanine, 1 g (Ala); with potassium, 1.2 meq (K), as KCl; or without potassium.

† Stand. dev. is listed below each avg value.

variation, and on the average is less than average values of 2.4 ml and 2.7 ml/g reported previously(10,11).

Summary. Changes in liver glycogen, water and potassium were compared in normal and potassium-deficient rats following administration of glucose, fructose and alpha-alanine, with and without potassium. Potassium-deficient rats deposited liver glycogen equally as well as normal rats. The simultaneous increases in liver water and potassium were likewise similar in the 2 groups of animals. No significant differences in changes in liver glycogen, water or potassium were noted with simultaneous administration of potassium.

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Serum Calcium Levels in Thyroid-Ablated Rats.* (24058)

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Radioiodine is used extensively to destroy the thyroid gland. Complete and permanent thyroid ablation can be achieved in rats with an I^{131} dosage of 3 to 4 μC/g body weight (1). Microscopic examination of peritracheal remnants from rats injected with I^{131} at this dosage revealed only superficial alteration of the intimately associated parathyroid glands, and for this reason the parathyroids have been presumed to be functional. At very high levels of I^{131} administration, however, enough of energetic beta radiation penetrates the parathyroid glands to effect an almost complete destruction(2). In a study of comparative reproductive capacities of female rats, whose thyroid glands had been destroyed with 5 μC/g of I^{131}, and rats whose thyroid and parathyroid glands had been removed surgically(3), it was brought to our attention that parathyroid deficiency might seriously affect experimental results(4,5). McLean and Urist(6) point out that parathyroid glands are of major importance in maintenance of normal blood calcium level. In the absence of parathyroid glands, the plasma calcium may fall 30% or more below normal. The calcium level in plasma should therefore be a good measure of the functional status of parathyroid glands. These studies compare the serum calcium levels of rats whose thyroids had been destroyed by low and high dosages of I^{131} beta radiation, with those in surgically thyroparathyroidectomized rats.

Methods. The animals used were female Sprague-Dawley rats obtained from the original colony. They were housed in metal cages on wood shavings and were fed Purina Laboratory Chow and tap water *ad lib.* When the animals were 60 to 80 days old, 12 were surgically thyroparathyroidectomized, 6 were given I^{131}† intramuscularly 5 μC I^{131}/gram body weight(1,3), 16 rats were given I^{131} intravenously 50 to 90 μC/g, and 12 normal rats of varying ages served as controls. Sixty or more days after treatment (see Table I for exact age at sacrifice) the animals were anesthetized with ether, and 6-ml blood sample was drawn into a heparinized syringe from the inferior vena cava. The red cells were separated from plasma by centrifugation, and plasma calcium was measured in duplicate 1-ml samples by the method of Rehell(7). When present, thyroid and (or) parathyroid remnants were dissected and fixed in Bouin's fluid. Paraffin embedded sections were cut at 6 μ and stained with hematoxylin and eosin.

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† I^{131} was obtained from Oak Ridge Nat. Lab. and was administered in basic solution of Na_2SO_3.

TABLE I. Serum Calcium Levels of I^{131} Thyroid-Ablated Rats and Surgically Thyro-parathyroidectomized Rats.

	Rats			Serum Ca^{++}
Treatment group	No.	Age (days)	Wt (g)	(mg/100 ml ± S.E.)
Control	12	143 to 443	292	9.84 .11
Surgery	12	472	259	7.86 .21
I^{131}, 5 μC/g	6	143	278	9.30 .02
" , >50 μC/g	16	300 to 400	185	7.91 .29

Underlined means were compared to the control mean by the t-test of Fisher(8) and P-value was beyond the 1% level of confidence.

Results. Serum calcium levels of various groups are shown in Table I. No significant difference was found between 8 control rats 143 days old and 4 controls 443 days old; therefore data for these groups have been combined. Rats that received I^{131} 50 μC/g had serum calcium levels identical to those given 90 μC/g of I^{131}; therefore these groups also have been combined. As shown in the Table, animals whose thyroid and parathyroid glands had been removed surgically or destroyed by very large amount of I^{131} had plasma calcium levels 22% below the normal mean. On the other hand, the low-level I^{131}-treated rats whose thyroid glands had been completely ablated, as shown in Fig. 1 (see also ref. 1), had a mean serum calcium level that differed only slightly from that of intact rats. For comparison the extent of parathyroid destruction following a massive dose of I^{131} is shown in Fig. 2.

Discussion. These measurements indicate that at relatively low dosages, 5 to 10 μC/g body weight, I^{131} can be used to effect complete destruction of thyroid gland of the rat

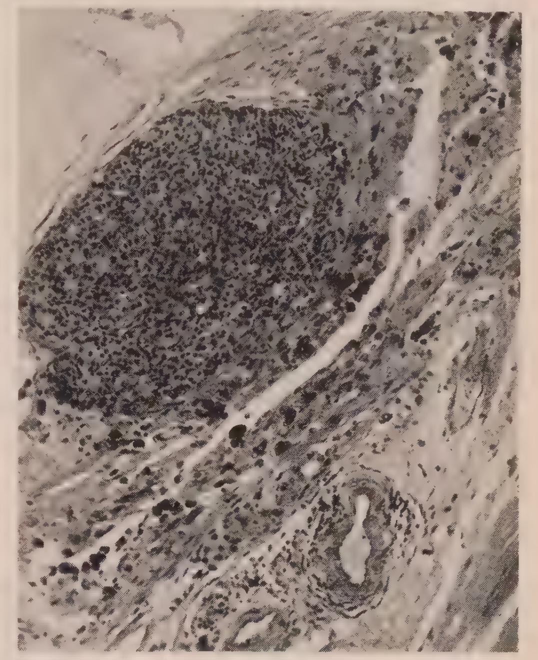

FIG. 1. Tracheal remnant from a rat 10 mo after receiving 10 μC/g of I^{131}. Note absence of any thyroid tissue and almost intact parathyroid gland. H and E, × 50.

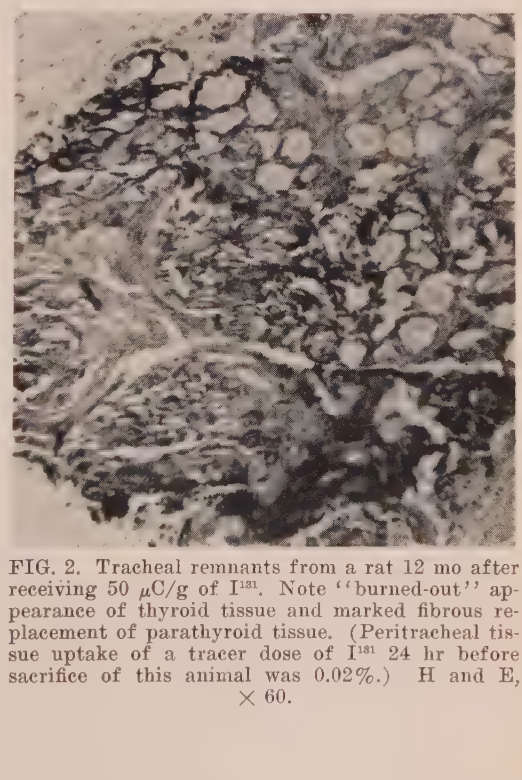

FIG. 2. Tracheal remnants from a rat 12 mo after receiving 50 μC/g of I^{131}. Note ''burned-out'' appearance of thyroid tissue and marked fibrous replacement of parathyroid tissue. (Peritracheal tissue uptake of a tracer dose of I^{131} 24 hr before sacrifice of this animal was 0.02%.) H and E, × 60.

without serious impairment of the capacity of parathyroid glands to maintain a normal plasma calcium level. At higher I^{131} dosages, however, the parathyroid glands are severely damaged, and the plasma Ca^{++} falls to the same level as that observed in surgically thyroparathyroidectomized animals.

Summary. Serum calcium levels have been measured in adult female Sprague-Dawley rats whose thyroid glands had been removed either with I^{131} or by surgery. The mean serum Ca^{++} level of rats whose thyroids had been completely ablated with 5 μC/g body weight of I^{131} was within normal limits, 9.3 ± 0.02 mg/100 ml. Complete destruction of the parathyroids, as well as the thyroid gland, was effected by a dosage of 50 $\mu C/g$ I^{131}.

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Role of Adrenaline and Nor-Adrenaline on Response of Cold-Acclimatized Animals to Chlorpromazine. (24059)

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Adrenaline has proved important in increased potential for chemical regulations observed in cold-acclimatized animals(1-7). Recently Hart(8) and Sellers(9) summarized present knowledge on acclimatization to cold in homeotherms. In terms of survival, acclimatization may be defined as an acquired increase in resistance to hypothermic effect of cold environment. Recently we reported that acclimatized animals are not only resistant to hypothermia caused by cold temperatures but also to that induced by chlorpromazine(10). The present study was designed to find out if decreased hypothermic effect observed in acclimatized animals after chlorpromazine injection was related in some way to adrenaline or nor-adrenaline action.

Methods. White male rats weighing 200 g were used. Thirty rats, acclimatized to cold for 4 weeks at 40°F were divided into 5 groups which were treated respectively with: (1) chlorpromazine (CPZ) (10 mg/kg); (2) CPZ (10 mg/kg) + adrenaline bitartrate (0.2 mg/kg of base); (3) CPZ (10 mg/kg) + nor-adrenaline bitartrate (0.2 mg/kg of base); (4) CPZ (10 mg/kg) + dibenamine (20 mg/kg) and (5) CPZ (10 mg/kg) + pyribenzamine (10 mg/kg). Five groups of control animals, non-acclimatized to cold, were treated in the same manner. All drugs were injected intraperitoneally. Rectal temperatures were measured with a thermocouple inserted at a depth of 40 mm. The cold-acclimatized animals after being in the cold for 4 weeks were kept for 24 hours at 72°F prior to drug injection. The experiment was performed in room maintained at 72°F.

Results. Fig. 1 illustrates the typical CPZ hypothermia which is not, however, significantly affected by injections of either adrenaline or nor-adrenaline. The potentiation of CPZ hypothermia by dibenamine and pyri-

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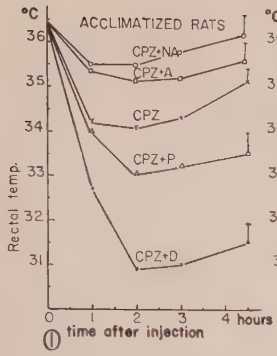

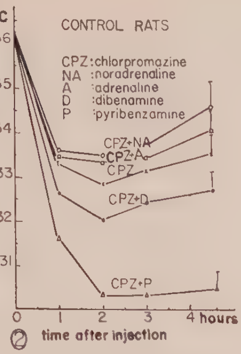

FIG. 1. Hypothermic responses to chlorpromazine, adrenaline, nor-adrenaline, dibenamine and pyribenzamine in rats acclimatized to cold (6°C) for 4 wk. Stand. error is indicated for each group.

FIG. 2. Hypothermic responses to chlorpromazine, nor-adrenaline, dibenamine and pyribenzamine in non-acclimatized animals. Stand. error is indicated for each group.

benzamine is of the same magnitude as the one reported recently(11). Results shown in Fig. 2 when compared with those shown in Fig. 1 confirm our previous findings(10). Indeed acclimatization to cold alleviates some of the hypothermic effect of CPZ. Also in Fig. 2 it is shown that both adrenaline and nor-adrenaline prevent to some extent the hypothermia of CPZ in acclimatized animals. In acclimatized and control animals both pyribenzamine and dibenamine potentiate the hypothermia of CPZ. In the acclimatized animals dibenamine is much more potent than pyribenzamine in that respect. In non-acclimatized animals pyribenzamine potentiates the CPZ hypothermia much more than dibenamine. It is also interesting to note that the antagonistic effect of acclimatization with regard to the hypothermia of CPZ is completely abolished by dibenamine.

Discussion. Hsich and Carlson(1) have shown recently that the increase in oxygen consumption caused by sympathomimetic substances is larger in acclimatized animals. It is possible then that acclimatized animals have become sensitized to adrenaline and nor-adrenaline. This may be the reason why these two substances antagonize the hypothermic effect of CPZ in the acclimatized animals but not in the control group (Fig. 1, 2). The fact that an adrenergic blocking agent, in this case dibenamine, completely abolished the antagonistic effect of cold-acclimatization on CPZ hypothermia, confirms this hypothesis. On the other hand it could be said that if cold-acclimatized animals are sensitized to adrenaline, they are desensitized to histamine since an antihistamine was less effective in potentiating CPZ hypothermia after acclimatization. Confirming this conclusion is the finding reported recently that acclimatization to cold prevents the thermic response to histamine(10). This conclusion was also reached in a previous study on systemic adaption of humans to a local cold stress(12).

Summary. The antagonistic effect of acclimatization on CPZ hypothermia is associated with increased sensitization of tissues to adrenaline and nor-adrenaline. Although normally these substances have no effect on CPZ hypothermia, they potentiate the antagonism of acclimatization towards CPZ. Furthermore dibenamine completely abolished this antagonism. On the other hand results are presented suggesting that acclimatization to cold may also be associated with a desensitization to histamine.

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Chromatographic Studies of Rat Kidney Lysozyme.* (24060)

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Experiments in this laboratory indicated that lysozyme activity of some tissues, particularly kidney of rat, may be altered under conditions of hypophysectomy and hormone administration(1). The large inhibition of enzyme activity after administration of thyroid hormone involved a change of state of the enzyme(2). Because of the stability of egg white lysozyme (EWL), it appeared that the kidney enzyme might be a convenient system for biosynthetic studies. It was, therefore, necessary to investigate a procedure for purification of the enzyme. Ion-exchange chromatography of the enzyme from egg white has been reported(3,4). Isolations of lytic enzymes from rabbit spleen and spleen and kidney of dog by chromatographic technics have been carried out by Jollés and Fromageot(5,6). Another lysozyme from papaya latex has recently been isolated as the mercury derivative(7). This paper reports chromatographic studies of rat kidney lytic enzyme (KLE) by use of ion-exchange methods. A partial preliminary report of this work has appeared(8).

Methods. Lytic activity in crude extracts or eluate fractions was determined photometrically as previously described(9). *Micrococcus lysodeikticus*, cultured and prepared as before(10), was used as the test organism. Enzyme activity is expressed as μg equivalent to crystalline EWL (Armour) or in terms of specific activity where activity is based on Folin nitrogen(1). Protein content was estimated by use of the Folin-Ciocalteu reagent (11) or extinction at 280 mμ. Fresh rat kidneys were obtained from animals of Rutgers stock colony (male albino rats of Wistar strain weighing 200 to 400 g). These tissues were immediately frozen until ready for use. When extracts were to be made, the kidneys were allowed to thaw at 5°C for 30 minutes and teased free of all surrounding tissue. *Preparation of resin.* Two volumes of water were added to the unused resin and the suspension immersed in boiling water bath 5 minutes. The resin was centrifuged and washed with water until the supernatant fluid remained clear. The resin was washed successively with N HCl, water and N NaOH. NaOH was decanted and a slurry was made by adding water. To increase rate of eluant flow through Amberlite IRC-50 (XE-64), the larger particles were separated by sedimentation. The slurry was poured into 1000 ml graduated cylinder and stirred vigorously. After particles settled for 7 min., the supernatant was removed by siphon. This process was repeated several times. The larger particles were then recycled with acid, water and base and buffered to pH 6.4 with 0.2 M phosphate buffer. A column was packed by gravitation and the bed measured 1.2 x 57 cm in all experiments. The same resin was used for all work reported here. Ten ml fractions were collected using an automatic fraction collector. Concentrations of cytochrome c have been expressed as μg equivalents to standard solutions of oxidized beef heart cytochrome c(12). (Wyeth and Co.) Absorption of reference cytochrome c at 415 mμ was linear with concentrations from 0 to 0.2 mg/ml. This wavelength was selected to increase the sensitivity of the assay. Closed-strip paper electrophoresis (200 volts; field strength about 4 v/cm; 8 to 15 hrs) was carried out with Whatman No. 3 paper strips (3 x 52 cm) with 0.2 M phosphate buffer at pH 6.4 as conductor, using CCl_4 as insulator in a 20°C chamber. The apparatus was similar in principle to that described by Markham and Smith(13). Densitometry was carried out according to Block, *et al.*(14) using a Photovolt Corporation densitometer. *pH gradient elution* was carried out using an apparatus which was essentially a modification of a mixing system described by Bock and Ling(15). This apparatus consisted of two rectangular chambers, a reservoir and a mixing chamber. A constant volume was maintained in the mixing chamber throughout the elution. The column with the buffered resin was first connected to the mixing chamber (closed system). The reservoir contained 500 ml 0.2 M NaH_2PO_4 at pH 4.5 while the mixing chamber held 500 ml 0.2 M Na_2HPO_4 at pH 9.3. The elution was carried out so that the drop rate from the column was 4 drops per minute and the volume in the mixing chamber was maintained at 500 ml. The enclosed system was under air pressure which was kept constant by a capillary device (T tube) immersed in a column of mercury. Direct elution was carried out with an identical column by standard procedures. The dropping rate was maintained at 4 drops per minute for which no air pressure was required.

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* Supported in part by res. grant (E-1119C) from the Nat. Inst. of Allergy, N.I.H., U.S.P.H.S. Paper of Journal Series, N. J. Ag. Exp. Sta. Abbreviations used in this paper are: EWL, egg white lysozyme (Armour); KLE, rat kidney lysozyme, and EEWL, equivalents of egg white lysozyme (Armour).

Results. Effects of pH upon KLE activity. Initial fractionations were made from chilled, 1:6 aqueous homogenates of thawed kidney tissue. After adjusting the pH of aliquots of the homogenate to different values, the fractions were centrifuged at 20,000 x g for 20 min. at 0°C. The clear supernatants were readjusted to pH 5.0, which appeared to be the pH of maximum stability of the enzyme, and assayed for activity(9) and Folin-Ciocalteu reaction. The pH values of 4-4.5 yield the greater specific activities. When a homogenate was heat-treated (90 seconds at 100°C) prior to centrifugation, the pH range of maximum stability was found to be 4.5-5.. Aqueous homogenates, adjusted to pH 4.5 ± 0.2 could be immersed in a boiling water bath for 90 seconds with negligible inactivation of the enzyme. This pH range was also effective for supernatant preparations. The pH optimum for supernatant enzyme stability was 5.0-5.5 during heating at 38°C for 90 minutes. The optimal buffer pH for the assay system after lysis was 6.2 which is similar to results previously reported for EWL(16). The starting preparation for chromatography experiments was subsequently obtained by heating a 1:6 aqueous homogenate at pH 4.5 for 90 seconds at 100°C, centrifuging and retaining the supernatant and one washing with an equal volume of H_2O. The combined supernatant and washings were dialyzed for 24 hours at 5°C against distilled water. *Volume reduction.* After removal from the dialysis tubing, the dialysate was adjusted to pH 5.0 and the volume was reduced by blowing air on a dialysis sac containing the enzyme at 5°C. The volume was reduced to 5-8 ml in this manner. Some loss of KLE activity inside the sac was experienced due to leakage of KLE through the membrane. A clear, pink solution was obtained which was then assayed and applied to the buffered column. *Gradient elution analysis* of 3 mg equivalents of EWL is shown in Fig. 1. Three peaks of enzyme activity have been resolved by this procedure. Absorption at 280 mμ and 415 mμ show peaks of inert proteins. A concentrated inert protein fraction was rapidly eluted. This gave rise to a large purification. The first peak contained a yellow-brown chromoprotein, probably similar to that seen by Pâleus and Neilands(17) with beef heart. The inert fractions also reacted strongly with the Folin reagent and absorbed strongly at 415 mμ. The 2 peaks of pigment material (415 mμ) in the region of the α and the first part of the β peaks of enzyme activity proved to be reduced

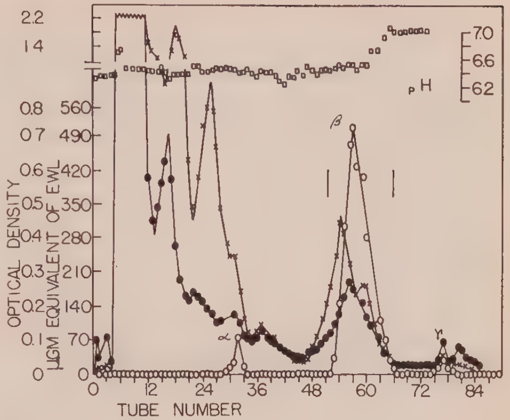

FIG. 1. Gradient elution chromatography of an extract of rat kidneys partially purified by heat coagulation and centrifugation, followed by dialysis and volume reduction. Open circles represent lytic activity expressed as equivalents of EWL. Solid circles represent optical density at 280 mμ (protein). Crosses represent optical density at 415 mμ (cytochrome pigments), and the open squares represent pH of tube contents. Each tube contained 10 ml eluate. Column load = 3.0 mg EEWL.

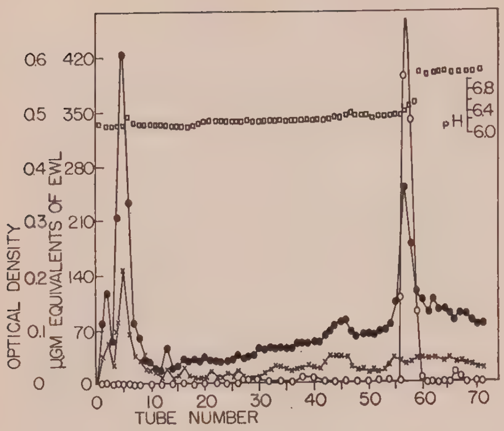

FIG. 2. Gradient elution rechromatography of the beta KLE peak of enzyme activity (tubes 52-66) shown in Fig. 1, which were combined, dialyzed and reduced in volume. Symbols are identical to those in Fig. 1. Column load = 500 μg EEWL. Each tube contained 10 ml eluant.

and oxidized forms of cytochrome c similar to the observations of Pâleus and Neilands(17) with beef heart cytochrome c. The eluant pH was alkaline during elution of the inert protein fraction, however, pH 6.4 ± 0.3 was maintained during elution of the enzyme activity up to tubes 58-60 where a rise in pH occurred. Fig. 2 shows a gradient elution rechromatography of the β peak of KLE activity. The contents of tubes 52-66 were combined, dialyzed and reduced in volume before rechromatography on freshly regenerated resin. By rechromatographing the major peak of enzyme activity, further purification of the enzyme is obtained signified by elution of inert protein in tubes 1-10. The major peak of enzyme activity is relatively free from cytochrome c and an overall purification of enzyme specific activity of about 100-fold has been achieved. Closed-strip paper electrophoresis of combined cytochromes and 3 peaks of enzyme activity failed to separate these components (mobility = 4 x 10^{-5} cm^2 v^{-1} sec^{-2}). Densitometry was carried out before color development to assay the pigments and after bromphenol blue development for total protein. Frequent tailing of basic proteins has been avoided using the closed-strip method.

Fig. 3 shows results of a direct elution of KLE extract using 0.2 M phosphate buffer at pH 6.4. Three peaks of KLE activity were also resolved by this procedure (Fig. 3A). The β peak represents about 30 times the activity of the α or γ peaks. In general, 75 to 85% of total original activity was recovered. Fig. 3B shows the Folin reaction. It was evident, since the Folin reaction produced greater equivalents than activity measurements, that rechromatography might result in further purification as was found in the gradient elution procedure (Fig. 2). In Fig. 3C, 4 pigments have been partially resolved compared to 2 pigment peaks from gradient elution analysis. This demonstrates a greater resolving power of direct elution analysis. These are also indicated by the Folin reaction in 3B. Each peak was in the oxidized form. The visible absorption spectrum for each peak has been measured (reduction of pigments by sodium hydrosulfite); maxima = Cl oxidized, 410, and 530 mμ; Cl reduced, 415, 520 and 550 mμ; C2 oxidized, 410 and 530 mμ; C2 re-

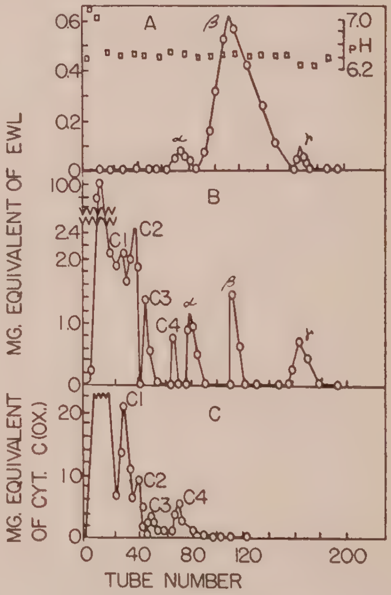

FIG. 3. Direct elution of supernatant, heat-treated, crude KLE. Column load = 13 mg EEWL. A. KLE activity (open circles) and eluant pH (open squares). B. Folin reaction for protein. C1, C2, C3 and C4 correspond to cytochromes; α, β and γ peaks correspond to KLE. C. Equivalents of beef heart cytochrome c measured by extinction at 415 mμ. C1, C2, C3 and C4 correspond to Folin protein peaks in B. Each tube contained 10 ml eluant. Fraction size = 10 ml. Column bed = 53 × 0.9 cm. Buffers as indicated in text.

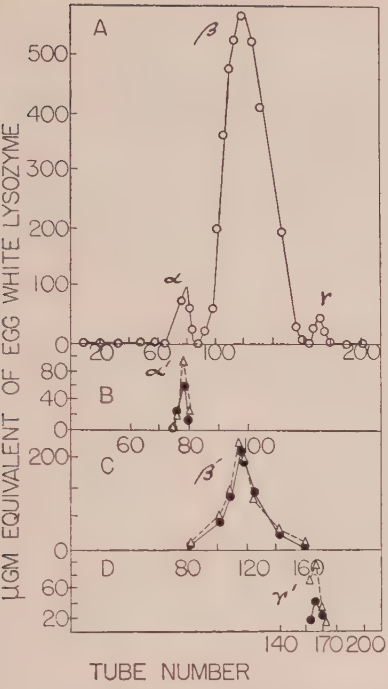

FIG. 4. Separate rechromatography by direct elution of individual KLE peaks obtained from a direct elution experiment. A. KLE activity of a direct elution experiment. Column load = 13 mg EEWL. B. Rechromatography of α KLE activity (solid circles) and protein measured by extinction at 280 mμ (open triangles). Column load = 352 μg EEWL. C. Rechromatography of β peak of KLE (symbols as in B). Column load = 7.72 mg EEWL. D. Rechromatography of γ peak of KLE (symbols as in B). Column load = 48 μg EEWL. Each tube contained 10 ml of eluant.

duced, 415, 520 and 550 mμ; C3 oxidized, 409 and 528 mμ; C3 reduced, 417, 520 and 550 mμ and C4 oxidized, 409 and 525 mμ. While comparisons have not been made on an equiprotein basis, some qualitative differences in the shapes of the curves, particularly in the ascending portion of the first peak, were seen. Previously, separation of KLE from pigments by classical fractionation or dialysis procedures was not obtained.

Separate direct elution rechromatography of each enzyme peak is shown in Fig. 4. Figs. 4B, 4C and 4D show separate rechromatography of each peak. Tube contents representing each peak were combined, dialyzed and reduced in volume prior to rechromatography. Protein was estimated by absorption at 280 mμ. The α peak appeared to contain more protein than measured by enzyme activity. This was due to a small amount of pigment which remained with this fraction. The β fraction was highly purified and probably free of pigment. The γ fraction may have contained more tyrosine or tryptophan than EWL or was still contaminated, to explain the higher values obtained from extinction measurements. Occasionally a very small fourth peak has been observed, however, the KLE concentration of such a fraction always fell at the lower limit of the enzyme assay.

Discussion. By direct or gradient elution analyses, 3 peaks of KLE activity have been resolved. Each of these peaks, found after direct elution, appears not to be an artifact since rechromatography of each peak under identical conditions produced peaks with similar chromatographic properties. The pH values of eluted fractions were very similar in gradient or direct elution experiments. During elution of the inert chromoprotein fraction, the pH rose sharply regardless of method of elution. The increase in pH seemed to be proportional to concentration of the inert fraction. After the chromoprotein fraction had been eluted, pH dropped to 6.4 and remained in the range of 6.4 ± 0.3 during elution of KLE activity until about 600 ml where the pH began to rise again.

While the presence of cytochrome c has not been reported for lysozymes isolated from other sources including the dog kidney, it has been found to occur with KLE rather consistently during several modes of fractionation. KLE and cytochrome c exist in free form and are difficult to separate because of strikingly similar properties, however, a separation may be accomplished by the methods described.

Summary. Chromatography of rat kidney lysozyme with Amberlite XE-64 ion-exchange resin by direct and gradient elution procedures have been described. Three peaks of enzyme activity have been resolved by either procedure. Separate rechromatography of each peak by direct elution substantiated that none of the peaks was an artifact. The central peak contains the major portion of enzyme activity and after rechromatography the enzyme is spectrophotometrically free of cytochrome c. Cytochrome c appeared, after direct elution, as 4 peaks

closely associated with the enzyme due to similar physical-chemical properties. Two peaks of reduced and oxidized cytochrome were found by gradient elution. Total purification of the specific enzyme activity was about 100-fold over the original supernatant.

The author expresses his appreciation to Miss Valentina Wojciechowski for excellent technical assistance in determination of enzyme activity.

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Effects of Gaseous Ions on Tracheal Ciliary Rate.* (24061)

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Recent experiments have shown that (+) and (–) air ions have marked effects on ciliary rate and other properties of the mammalian trachea, both *in vitro*(3) and *in vivo* (4). It seems reasonable to conclude that these effects stem from physiologically significant alterations in state of gases present in ambient atmosphere and that deliberately produced changes in composition of this atmosphere would be reflected in the response of the tracheal tissue. The following experiments were performed to test this assumption.

Methods. Anesthetized rabbits were tracheotomized and placed in humid-air ionizing chamber according to technic already described(4). Gases used were warmed to 25-28°C, bubbled through water, and blown continuously into the chamber. Since earlier work(2) had shown that presence of smog or cigarette smoke in ambient atmosphere altered the action of air ions on bacterial suspensions, cigarette smoke was employed in some of these experiments. It was produced in mechanical device operating under negative pressure, and then treated in the same way as gases. For N_2 and CO_2 experiments it was necessary to sacrifice the rabbits with an overdose of nembutal; experiments with O_2 and cigarette smoke permitted exposure of living as well as sacrificed animals. As in previous work(1-4): (a) ions were generated by β radiation from sealed tritium foils, a rectifying circuit allowing selection of (+) or (–) ions, (b) the same stroboscopic method was employed to determine rate of ciliary beat (accuracy ± 50 beats/min.), and (c) atmospheric temperature in exposure chamber varied from 25-28°C on different days, while relative humidity remained close to 90%.

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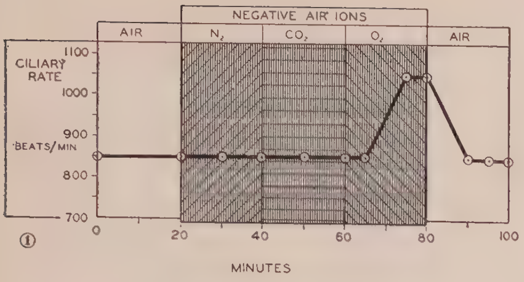
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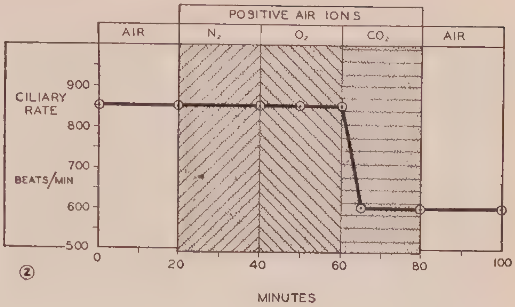
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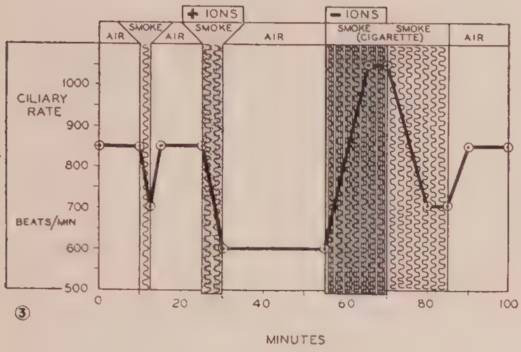
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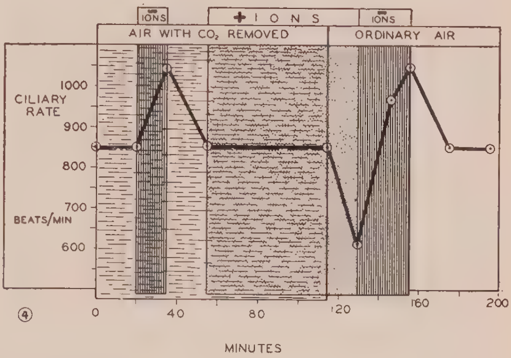
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FIG. 1. Action of negative ions on tracheal ciliary rate in N_2, CO_2, and O_2.

FIG. 2. Action of positive ions on tracheal ciliary rate in N_2, O_2, and CO_2.

FIG. 3. Action on tracheal ciliary rate of cigarette smoke, cigarette smoke and positive ions. cigarette smoke and negative ions.

FIG. 4. Action of positive ions in air with and without CO_2. (Negative ions were administered before and after the experiment to establish that the tissue was normally reactive.)

Results. When administered in unmodified humid air, (–) air ions increase ciliary rate by about 200 beats/min (reaching maximum effect within 10-20 minutes), while (+) air ions lower ciliary rate by about 300 beats/min or abolish ciliary activity altogether (reaching maximum effect within 15-20 minutes). These effects are seen both in tracheas of living animals and in extirpated tracheal strips(3,4). However, when commercially-produced gases or cigarette smoke were substituted for air. a number of deviations from the usual pattern were observed:

1. *Negative ions in gases and cigarette smoke.* (–) ions had no effect whatever on tracheal ciliary rate in N_2 or CO_2 (Fig. 1). In O_2 (Fig. 1) or in cigarette smoke (Fig. 3) (–) ions raised the ciliary rate to the same extent and at same rate as in ordinary air.

2. *Positive ions in gases and cigarette smoke* . (+) ions had no effect whatever on tracheal ciliary rate in N_2 or O_2 (Fig. 2). In CO_2 (Fig. 2) or in cigarette smoke (Fig. 3) (+) ions lowered ciliary rate more rapidly (within 2-5 minutes) than in ordinary air (within 15-20 minutes). When ordinary air was passed through a CO_2-absorber before reaching exposure chamber, (+) ions had no effect on ciliary rate; when CO_2-absorber was removed, (+) ions produced usual ciliary-rate depression at usual rate (Fig. 4).

3. *Gases and cigarette smoke alone.* N_2, O_2, or CO_2 by themselves had no effect on ciliary rate within the 20-minute exposure employed. Cigarette smoke alone, however, promptly decreased ciliary rate by about 200 beats/min. The decrease persisted only so long as cigarette smoke remained in the chamber. When the smoke was flushed out with fresh air, the ciliary rate returned at once to its initial level (Fig. 3).

These experiments make it clear that (–) ion effects on tracheal cilia can take place only when more than a certain minimum of O_2 is present. Evidently these effects proceed at maximal rate in ordinary air, and are not accelerated by 100% O_2, or diminished by additional components of cigarette smoke.

Analogously, (+) ions require more than a certain minimum of CO_2 to exert their effects. However, increases in CO_2 concentration above that of ordinary air do increase the rate at which (+) ions produce their effects.[†]

The agent in cigarette smoke that causes reversible lowering of ciliary rate is not known. Whatever it may be, its action is effectively neutralized by (–) ions, which raise the ciliary rate just as well in a heavy atmosphere of cigarette smoke as they do in fresh air.

On the other hand, the reversible drop in ciliary rate induced by cigarette smoke alone is quickly lowered still more and made permanent by (+) ions; "permanent" in the sense that the low rate will continue indefinitely after smoke and (+) ions have been removed, although it can still be reversed by (–) ions. The extent of the (+) ion lowering in cigarette smoke is the same as in ordinary air; the rate of lowering is 3-10 times faster in cigarette smoke. Whether this more rapid rate of action of (+) ions in smoke is due to increased CO_2 concentration alone, or involves other components of cigarette smoke as well, remains to be established.

In experiments performed in humid air about 1 x 10^9 ions/cm^2/sec, as measured with the Beckett probe and Keithley electrometer, impinged on tissue surface. Ion density measurements did not vary greatly from this figure when N_2, O_2, or CO_2 were substituted for air, but this does not signify that equivalent numbers of ions necessarily were produced from the various gases, for the ambient atmospheres were far from pure. Among sources of pollution were 1) air seepage into the plastic chamber, 2) impurities in the commercially-made gases which were bled from metal tanks, 3) water vapor purposely introduced to keep the tissue moist. Nevertheless, these gases did not seem to introduce any complicating factors, presumably because the physiologically active components (O_2 and CO_2) were present in quantities below their threshold levels.

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[†] In ordinary air, rate of (+) ion action is significantly higher *in vivo* than *in vitro*. (+) ion action mediated through additional CO_2 from expired air of living animal is evidently sufficient to cause this difference.

Infectious Canine Hepatitis Virus Studies with Special Reference to Passage of Raccoon Tissue Cultures. (24062)

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Previous reports have described growth of infectious canine hepatitis (ICH) virus in tissue cultures derived from dog kidney(1,2,3). More recently, it has been shown that ICH virus grows in tissue cultures of ferret and pig kidney origin(4). Green *et al.* reported transmission of fox encephalitis virus infection to 1 of 14 raccoons, *Procyon lotor*, by intracerebral injection, while raccoons given virus by intraocular route were routinely infected(5), indicating that certain cells within the ocular tissues and central nervous system of these animals support growth of virus. Recently it was shown that there are no serological differences between ICH and fox encephalitis viruses(6).

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This paper deals with: 1. Cultivation of ICH virus in tissue cultures derived from wild raccoons; 2. Isolation of ICH virus from kidneys of healthy dogs; and 3. Distribution of specific ICH antibodies in sera of dogs in rural areas of Kansas.

Materials and methods. Virus strain. The infectious canine hepatitis (ICH) virus used in this study was received from Dr. James A. Baker, Veterinary Virus Research Institute, Cornell University, Ithaca, N. Y. It was received as frozen spleen. The virus was passed once in antibody free puppies by intraperitoneal (IP) injection of 1 ml of 10% ground spleen in phosphate buffered saline. When the infected puppies were moribund, the spleens were harvested and frozen at –70°C until used. *Animals.* From 1953 to 1957, 216 puppies were bled by cardiac puncture. The sera were stored at –4°C until used. The age of puppies ranged from 3 to 7 months. Puppies were received from farms in Central and Northwest Kansas. The number of puppies/litter ranged from 3 to 9. Wild adult raccoons were obtained from Iowa, Nebraska and Florida. One collared Peccarey, *Tayassu angulatus,* was obtained from Texas. Other animals were obtained from local animal dealers. Raccoons were routinely bled at the time they were sacrificed for tissue culture studies. *Roller tube tissue cultures* were prepared by 2 methods. (1) Tissue cultures were prepared from explants of the tissue of choice or (2) animal kidneys were trypsinized according to the method of Younger(7). Trypsinized, packed kidney cells were used in final dilution of 1:400. All cultures were grown in 1 ml of medium #199(8) with 2% horse serum and 3% $NaHCO_3$. Penicillin and streptomycin were present at final concentration of 100 units and 50 mg/ml respectively. Growth medium was replaced on cultures 3, 4, or 5 days after preparation as indicated by a lowered pH. Viral inoculum was added to cultures after medium change without subsequent changes in culture conditions. All infected and control cultures were observed every other day for 7 days or until cytopathologic effects (CP) occurred. Virus infected tissue culture fluids were stored at either –4°C or –70°C. Four to 8 cultures were inoculated for each virus passage. Each tube was injected with 0.1 ml of virus fluid. Virus titrations were carried out in trypsinized raccoon or dog kidney cultures. Medium #199 buffered with $NaHCO_3$ was employed as diluent for both virus and serum. Infective end points were calculated by the Reed-Muench formula(9). *Standard ICH Immune Serum.* Puppies free of ICH immune antibodies by the neutralization test were injected IP with 1 ml of 10% ICH virus infected dog spleen. Four weeks later one survivor was bled and serum stored at –4°C until used. The ICH convalescent serum had a neutralizing titer of 1:5120 against 320 $TCID_{50}$ of the virus while the preinoculation serum had a titer of less than 1:2. *Serological procedures.* Identification of either ICH virus or specific ICH antibody in serum was made by neutralization tests. The titer of standard ICH immune sera was determined by mixing 2-fold falling dilutions of serum with 100-320 $TCID_{50}$ dog kidney tissue culture (DKTC) virus. The highest serum dilution preventing CP was designated as the titer. Assay of farm puppy sera for ICH antibodies was carried out by mixing 32-320 $TCID_{50}$ DKTC virus with either 1:2 or 1:5 final dilution of serum. In the ICH antibody assay of raccoon sera 10-100 $TCID_{50}$ DKTC virus was employed against final dilution of 1:5 serum. DKTC virus below the tenth passage level was employed for assay of all sera for ICH antibody. Paired serial 10-fold dilutions of DKTC or raccoon tissue culture virus were prepared, the initial one being 1:5 dilution. Suitable dilutions of virus were mixed with equal volumes of preinoculation and convalescent sera in final dilution of 1:2 and 1:10 respectively. All serum-virus mixtures were agitated and incubated at room temperature for 1 hr. Each mixture was inoculated into at least 2 tissue culture tubes in a volume of 0.1 ml each. All sera were heated at 56°C for 30 minutes just before use. Virus titrations were always carried out simultaneously with each neutralization test. Uninoculated and serum control cultures were always included in the test. Cultures were observed daily for 7 days or until

TABLE I. Results for Neutralization of 4th Passage ICH Virus in Various Raccoon Epithelia.

Standard serum (dog)	Source of epithelium			
	Kidney	Corneal (endothelium)	Ventrical (ependymal)	Bladder (transitional)
Normal	3/3	3/3	3/3	3/3
Immune	0/3	0/3	0/3	0/3

Numerator = No. of tubes showing cytopathology. Denominator = No. of tubes inoculated.
4th passage raccoon kidney virus titered $10^{-4.5}$/ml; other tissues not titered.
Final dilution of normal and immune sera was 1:2 and 1:10, respectively.

CP occurred. *Results. Passage of ICH virus.* Dog kidney explant roller cultures were each inoculated with 0.1 ml of 10% emulsion of dog spleen infected with ICH virus. Widespread CP occurred in cultures on 4th and 5th days. The fluids were harvested, pooled and passed a second time. CP was apparent on 3rd day and complete on 4th day. In this manner DKTC virus was serially passed 12 times. The next 6 passages were achieved in trypsinized roller tube cultures with similar results. The 18th passage DKTC virus constituted the inoculum for initiation of the raccoon kidney tissue culture (RKTC) virus passage series. Cultures of raccoon *kidney explants,* scrapings from urinary bladder, corneal endothelium and brain ventricle ependymal cells were inoculated with 1:10 dilution DKTC virus. Widespread CP was present in all cultures in 4 days. The second to 4th passages were carried out in a similar manner with the same result. The 4th passage series of cultures were prepared in duplicate and inoculated with serum-virus mixtures of normal and standard immune dog serum. The results in Table I show that the cultures derived from different raccoon tissues all support growth of raccoon tissue culture virus. CP produced in these cultures by ICH virus was similar to that previously reported(1) for dog kidney cultures. *RKTC virus* and bladder passage virus were serially transferred to 40th and 7th passage levels respectively. Bladder virus in 7th passage with titered 10^4 $TCID_{50}$ ml in DKTC was discontinued. Results for comparative infective titers of RKTC and DKTC viruses in raccoon and dog kidney cultures are illustrated in Table II. They show that there are no significant differences in the amounts of either virus produced in either type of culture. Other tissue cultures of rabbit and hamster kidney and bladder epithelium; fetal bovine kidney, tongue and bladder epithelium, mouse mix (kidney, spleen, liver, lung), peccarey, monkey and fetal pig kidney were inoculated with RKTC virus below 18th passage level. On the 4th day after inoculation, the peccarey and fetal pig kidney cultures all showed widespread CP whereas controls remained unchanged throughout 7-day observation period. No apparent CP occurred in any of the other cultures even when they were incubated for additional 7 days. Second passages were carried out with the same results. Peccarey and fetal porcine kidney virus passages were discontinued. *ICH Isolates.* During this research, a characteristic CP appeared spontaneously in uninoculated trypsinized dog kidney cultures prepared from 2 different dogs. In the first instance, the puppy had no known history of exposure or disease. The second dog had been exposed to ICH disease 3 months before its kidneys were removed and trypsinized. The incidence of spontaneous CP in cultures was one in every 15 tubes examined. Standard immune dog serum and serum of the first dog in final dilu-

TABLE II. Results for Titration of Tissue Culture ICH Virus of Raccoon and Dog Kidney Origin in Raccoon and Dog Kidney Cultures.*

RKTC virus			DKTC virus		
	Substrate			Substrate	
Pass.	RK	DK	Pass.	RK	DK
8	$10^{-4.5}$	$10^{-4.5}$	3		10^{-4}
9	"	10^{-4}	9	$10^{-4.5}$	10^{-5}
14	NT	"	17	$10^{-4.0}$	10^{-5}
19	10^{-4}	NT	18	$10^{-4.5}$	$10^{-4.5}$
25	$10^{-4.5}$	"			
34	10^{-5}	"			
40	10^{-4}	10^{-4}			

* RKTC, DKTC = Raccoon, dog kidney tissue culture virus. RK, DK = Raccoon, dog kidney cultures.

NT = Not tested.

TABLE III. Results for Presence of Neutralizing Antibodies against ICH Virus in the Sera of Puppies and Raccoons.

Serum source	Age (mo)	No. sera	Serum antibodies* Positive	Negative
Raccoons	Adult	23	1	22
Puppy	3–7	216	17	199

* Dog sera tested in a final 1:5 dilution or less against 32-320 $TCID_{50}$ virus. Raccoon sera tested in a final 1:5 dilution against 10-100 $TCID_{50}$ virus. DKTC virus used in all tests.

tion of 1:10 neutralized at least 1000 $TCID_{50}$ of homologous ICH isolate and DKTC virus. Serum of second dog was not tested for virus neutralization.

Results for serum survey for ICH antibodies. Neutralizing substances were found in serum of 1 of 23 raccoons against 10 to 100 $TCID_{50}$ DKTC virus. The results shown in Table III indicate under the limited number of sera tested, that less than 5% of raccoons show evidence of ICH infections. Less than 9% of sera from farm puppies tested showed presence of specific antibodies against ICH virus.

Final identification of the RKTC virus at 39th passage level was by direct neutralization with standard immune serum and the inoculation of antibody-free puppies with virus.

Results for the serum-neutralization test showed that standard normal dog serum in final 1:2 dilution failed to neutralize RKTC virus while specific immune serum in final 1/10 dilution neutralized at least 4 logs of the virus.

Nine antibody-free puppies were divided into 2 groups. Each of 5 puppies in the first group were injected intramuscularly with 0.5 ml of RKTC virus. The second group served as contact controls and were housed with puppies in Group 1. In the first group temperature elevation of 102.2°F or above commenced 2-4 days after inoculation, persisted 9-15 days and then dropped below 102.2°F. Three puppies gained weight throughout 28-day observation period. The other 2 puppies developed unilateral corneal opacities, lost weight and 1 died on 19th day of infection. Convalescent serum was not obtained from this dog. Lesions pathognomonic for ICH disease were not found at autopsy. Severe bacterial pneumonia was found in both lungs and hemolytic streptococci and coli were recovered from both liver and spleen on blood agar plates. It was concluded that this dog did not die of ICH disease.

In the second group temperature elevations above 102.2°F commenced after 12 days of contact with Group I, persisted for 3-11 days, and returned to 102.2°F or lower. All puppies gained weight and remained essentially normal throughout 28 days of observation. Temperature elevations in either group of puppies did not exceed 104°F. Persistence of temperature elevations in the 4 puppies in Group II was 4-6 days less than the inoculated puppies in Group I indicating that ICH infection in these dogs was more mild than in Group I.

Results of serum-neutralization studies for 9 preinoculation and 8 convalescent dog sera against DKTC virus showed that specific ICH antibodies appeared in convalescent sera of both groups. The 9 pre-inoculation sera were negative for ICH antibodies in final dilution of 1:2 against 320 $TCID_{50}$ DKTC virus while the convalescent sera in final dilution of 1:10 neutralized at least 3 logs of virus. Appearance of circulating antibodies in blood of puppies in Group II established the transmission of virus by direct contact from puppies in Group I with subsequent development of inapparent ICH infections.

In other studies, it was shown that dogs recovered from infection with RKTC virus and their contact controls were solidly immune against virulent challenge spleen virus while 30% of the non-immune controls died.

Summary. 1. Infectious canine hepatitis (ICH) virus grown and passed 39 times in tissue cultures derived from wild raccoons. The tissue culture virus in 39th passage was neutralized by specific immune serum prepared against the original dog spleen virus. When the 39th passage virus was introduced into antibody free puppies, all developed a mild ICH infection resulting in appearance of specific serum neutralizing antibodies in blood against dog kidney tissue culture virus. 2. Eight percent of the puppies, 3-7 months of age, obtained from farms in Central and Northwest Kansas, have specific neutralizing antibodies against ICH virus; ICH antibodies in a 1:5 titre were found in 1 of 23 sera from wild raccoons. 3. ICH virus was isolated from dog kidney tissue cultures derived from 2 apparently healthy dogs. In one instance, specific ICH neutralizing antibody was present in the blood.

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Antagonistic Action of Liver Amino Acids and 2,4-Dinitrophenol on Growth of *Escherichia coli*. (24063)

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It is known that extracts and fractions of liver protect animals from toxic effects of poisons and hormones. Ability of liver extracts to counteract the toxic activity of thyroid powder and 2,4-dinitrophenol (DNP) on various living systems is an example of this type of antagonism and is of particular interest because DNP and thyroxin are both inhibitors of oxidative phosphorylation. Crude liver preparations and components of liver extracts reverse thyroid stress occurring in rats fed with purified diets containing thyroid powder or iodized protein(1). Attempts have been made to identify the unknown factors (2,3) responsible for this anti-thyrotoxic activity, or to refer such activity to known components of the liver(4,5). An antagonism of liver extracts against inhibitory action of DNP has been described in studies with mammalian tissue homogenates(6), as well as with bacteria(7). Analysis of growth-stimulating and anti-DNP activity of liver extracts in bacterial systems shows that several fractions are involved: one is composed of bile salts; another of liposoluble vitamins; a third of steroids; a fourth of unsaturated fatty acids and possibly hydroxy-acids; and a fifth of amino acids and oligopeptides. The hypothesis has been expressed that these substances represent physiological antagonists of thyroid hormones in mammalian tissues, at a cellular level. Similar results were obtained by other authors(4,5) using metabolic-rate test of rats fed with iodized protein for titrating the anti-thyrotoxic activity of liver components. The present work is concerned with the amino acid fraction of liver extracts.

Technics. Liver-amino acid preparation was obtained as follows: Cow's liver, homogenized in Waring Blendor and autolyzed under toluene at 37°C, was dried, pulverized, and extracted by refluxing twice each with boiling ethanol followed by hot water. The residue of coagulated proteins was washed several times with H_2O and hydrolyzed with $6N$ HCl at 100°C for 18 hours. HCl was removed by vacuum distillation and the residue brought several times to dryness after addition of water. The dry material was then dissolved in H_2O. The resulting preparation had a nitrogen content of 13 mg/ml and was used without neutralization for chromatographic analysis, or was neutralized with KOH and sterilized for microbial assays. Medium A used for *E. coli* strain B had the following

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TABLE I. Action of DNP, Liver Amino Acids, and Mixture A on Growth of *E. coli*.

DNP, moles		Liver amino acid prep. (.1 ml)	Mixture A (.5 ml)	Growth (K.U.)*	
2×10^{-4}	4×10^{-4}			4 hr	8 hr
—	—	—	—	23	57
—	—	+	—	45	60
—	—	—	+	43	57
+	—	—	—	7	38
—	+	—	—	3	13
+	—	+	—	24	54
—	+	+	—	15	51
+	—	—	+	27	58
—	+	—	+	17	56

* Klett-Summerson Colorimeter, red filter, readings at time 0 subtracted from the subsequent readings, values of growth expressed in Klett Units.

Medium: Synthetic medium A, 4.3 ml; DNP 10^{-2} solution, .1 and .2 ml; liver amino acid prep., .1 ml; amino acid mixture A, .5 ml; dist. H_2O, up to 5 ml in each tube.

Inoculum: .1 ml *E. coli* strain B in synthetic medium A (2×10^9 cells/ml).

Growth: Static culture in sterile Klett tubes placed in water bath at 37°C.

composition: K_2HPO_4, 7 g; KH_2PO_4, 3 g; Na citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; glucose, 2 g; H_2O to 1 liter. DNP, added to this medium at levels of 2 x 10^{-4} M, gave respectively an inhibition of 80 and 90% in growth of *E. coli* strain B (Table I). Within 4 hours after inoculation, turbidity of the 5 ml culture had doubled in synthetic medium A containing 0.1 ml of liver-amino acid preparation, an amount which almost completely removed the inhibition of the 2 x 10^{-4} and 4 x 10^{-4} M levels of DNP (Table I).

A chromatographic analysis of liver-amino acid preparation has been accomplished with Whatman No. 1 filter paper and a bidimensional system consisting of butanol:acetic acid:water (6:25:25) and phenol:water (4:1) in an NH_3 atmosphere. Several chromatograms were run simultaneously by this technic, some being reserved for biological assays and others for development with ninhydrin (Fig. 1).

Anti-DNP activity of the spots was tested by placing dry undeveloped chromatograms on the surface of DNP-minimal agar seeded with the indicator organism. To prepare agar for the assay, 5 ml of *E. coli* strain B (10^9 cells/ml) was added to 500 ml of synthetic medium A containing 1.8% agar and 10^{-4} M DNP; the preparation was poured immediately onto an 11 by 17 inch sterile plate and allowed to solidify. After 30 minutes at room temperature, the chromatogram sheet was removed and the plate incubated at 37°C for 24 hours. At the level of 10^{-4} M, DNP inhibited completely the growth of *E. coli* strain B in solid medium, but some growth appeared on the surface of the agar, in locations corresponding to the position of migrated amino acids. Growth of the indicator strain, however, was not restricted to well-defined spots corresponding to those found in a ninhydrin-developed chromatogram; confluent zones of growth were seen, presumably arising from additive or synergistic action produced by diffusion and mixture of single eluted components.

In other chromatograms, the position of each amino acid was identified by means of ultraviolet lamp, and the spots were eluted and tested for growth-stimulating and anti-DNP activities in the synthetic medium A. Most of the eluted components showed no activity, a few (glutamic and aspartic acids, arginine, isoleucine, and cysteine) exhibited a slight degree of antagonism to DNP; the same negative results were obtained by testing activity of commercial, pure amino acids. It was concluded, therefore, that no single amino acid, but only a mixture of amino acids,

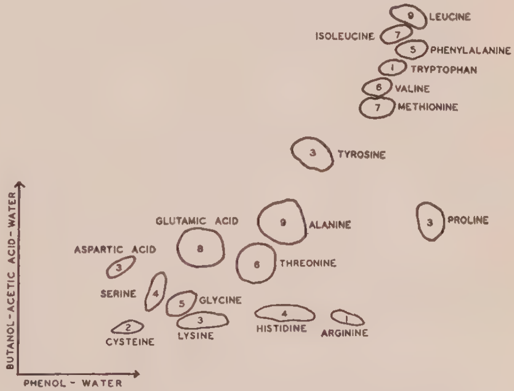

FIG. 1. Ratio of amino acids in chromatograms of liver preparation. In Mixture A, the amino acids have the above ratios, on a molecular basis. In Mixture B, 2 moles of the following amino acids have been added to Mixture A, giving a total of 100 moles: methionine, tyrosine, histidine, cysteine, tryptophan, ornithine, asparagine.

has growth-stimulating and anti-DNP activity; this is in accord with results obtained with undeveloped chromatograms on bacteria-seeded agar.

Quantitative composition of the liver hydrolyzate was established by evaluating intensities of the ninhydrin-developed spots of amino acids in bidimensional chromatograms; ratios of the component amino acids are shown in Fig. 1. A mixture of amino acids, having the reported ratios, was prepared with commercial products (Mixture A). The level of amino acids and the nitrogen content in the mixture were equal to one-fifth of corresponding values of liver hydrolyzate. A 0.5 ml quantity of this mixture showed almost the same growth-stimulating and anti-DNP activity as the corresponding amount (0.1 ml) of liver hydrolyzate (Table I).

Addition of several components of liver extracts (bile salts, lipo-soluble vitamins, steroids) to Mixture A did not enhance the anti-DNP activity of the amino acid combination on growth of *E. coli* strain B. Some increase was obtained by adding to Mixture A a supplement of amino acids destroyed during the process of acid-hydrolysis (tryptophan, tyrosine, histidine, cysteine, methionine) (Mixture B).

Discussion. Several questions arise in assessing the significance of these findings. First it should be observed that the ratio and quantitative relationship of amino acids shown to have anti-DNP activity may have no particular specificity as opposed to other ratios. Furthermore, there is no reason to suppose at present that liver as an amino acid source is superior to any other mammalian tissue. Since it is well known that many poisons and inhibitory agents act at lower concentrations in the absence of organic substances, there is the possibility that anti-DNP activity in bacterial systems is merely another example of this widely recognized phenomenon. Basic amino acids would be expected to combine extra-cellularly with DNP and reduce its effective concentration. However, additional experiments, not reported here, show that the amino acid preparation will allow induced-enzyme synthesis in DNP-inhibited cells after removal of extra-cellular DNP. Glycine or single amino acids do not have this property. Furthermore, by using cysteine-resistant *E. coli* mutants as test organism, it has been shown that anti-DNP activity obtained with small amounts of cysteine does not increase with amount of amino acid added. Such an increase would be expected if cysteine were acting solely by extra-cellular binding of DNP in proportion to the amount of reactants present.

Summary. 1) Liver hydrolyzates counteract the inhibitory action of 2,4-dinitrophenol (DNP) on both mammalian cells and bacteria. Of the several fractions responsible for activity of liver extracts, one is composed of amino acids. 2) Growth-stimulating and anti-DNP activity of this fraction has been tested on *Escherichia coli* strain B, and its composition established by chromatographic analysis. No one of the single components has activity, but a mixture of amino acids stimulates growth-rate of *E. coli* strain B in synthetic medium and counteracts inhibition by high concentrations of DNP. 3) A mixture of commercial amino acids has been prepared, having similar composition and activity to liver-amino preparation.

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Influence of Sex of Hypophyseal Gland on Mammary Cancer Development in Mice.* (24064)

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It has been previously demonstrated that castrate male mice bearing transplanted ovaries have a higher incidence of mammary cancer than intact females(1). This observation has been confirmed in mice of the A stock and in AZ F1 hybrids, although in mice of this latter stock the increased susceptibility in castrate males with ovaries was indicated only by an earlier cancer age(2,3). The fact that castrate males with transplanted ovaries and a piece of vaginal tissue exhibited signs of continuous estrogenization led these investigators to postulate that the greater susceptibility to mammary cancer in these animals might be due to a continuous ovarian stimulation from the male pituitary "in such a way that at all times sufficient ovarian estrogen is produced by the grafted ovaries"(2). Recently(4) taking advantage of a surgical technic allowing transplantation of functional hypophyseal gland tissue in hypophysectomized mice, studies on the estrous behavior of castrate male mice with grafted ovaries and either female or male pituitaries, demonstrated that under these conditions these animals exhibited continuous ovarian stimulation regardless of the sex of the pituitary gland they carried. Therefore it was concluded that the continuous non-cycling type of gonadotropic stimulation observed in males was not dependent on the sex of the pituitary *per se* but rather in some other controlling structure. On the basis of these observations it was considered of interest to determine mammary cancer incidence and average cancer age in castrate male mice with transplanted ovaries and either male or female pituitaries. If the conclusions drawn from our previous experiments were correct, the same incidence of breast cancer and average cancer age would be expected to occur in castrate male mice with transplanted ovaries and either male or female pituitaries. In addition, the higher cancer susceptibility in males as compared to that in females would be maintained regardless of the sex of the pituitary glands the males might carry. Data supporting these hypotheses are here presented.

Method. Female and male F1 hybrid mice resulting from the cross between A ♀ x Z ♂ were used. When approximately 6 weeks of age the females were divided into 2 groups one of which was submitted to bilateral ovariectomy followed by the subcutaneous graft of their own ovaries into the axillary region and the other kept as sham ovariectomized controls. All males were castrated and subsequently transplanted with 2 ovaries taken from donors of the same strain and age. One week after operation they were divided into 2 groups. One was submitted to total hypophysectomy followed by transplantation of male hypophyseal glands taken from isologous male donors of the same age. The remainder group was also subjected to total hypophysectomy followed by transplantation of female hypophyseal glands taken from isologous female donors of the same age. Hypophysectomy was performed according to the technic described by Thomas(5) modified by D. Ferguson (personal communication) and the grafting of the hypophyseal gland by the method described previously(4) consisting essentially in placing the entire gland to be grafted into the emptied *sella turcica* of the hypophysectomized host. By this procedure the over-all incidence of successful takes obtained in this experiment was 75% out of a total of 60 operated animals. Mice bearing successful hypophyseal grafts showed a sustained increase in body weight throughout their lives, reaching a mean value of 27 g 12 months after operation, whereas in those failing to accept the graft the body weight remained the same or decreased, with a mean terminal weight of 19 g. Following surgery

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mice were kept on the standard laboratory chow and examined twice weekly for appearance of palpable mammary tumors which were subsequently diagnosed histologically.

Results. The results can be summarized as follows: Of 41 sham ovariectomized non-breeding females, 34 or 83% developed mammary cancer at an average age of 479 ± 21 days. Of 22 ovariectomized females bearing their own ovaries grafted, all or 100% developed cancer at 404 ± 20 days. The difference between these 2 groups in regard to cancer incidence is not statistically significant whereas the difference in average cancer age is significant at the 5% level. Cancer incidence in hypophysectomized-castrate male mice with transplanted ovaries and male pituitaries was also 100% and average cancer age 342 ± 18 days. Roughly the same results were obtained in hypophysectomized-castrate male mice with transplanted ovaries and female pituitaries since also in this group all mice developed mammary cancer (100%) at an average cancer age of 327 ± 15 days. The slight difference observed in cancer age between both groups of males is not significant. However by comparing cancer age figures between males and females it is clear that the former developed cancer significantly earlier than the latter.

Discussion. The results listed confirm the observations made by Murray(1) and by Huseby and Bittner(2,3) in regard to the higher susceptibility to mammary cancer in castrate male mice with transplanted ovaries as compared to that in females. However the interpretation of this phenomenon by the latter investigators assuming that the greater susceptibility in males could be explained as due to the continuous non-cyclic ovarian stimulation by the male hypophysis does not seem to agree with our findings. The fact that castrate males with ovaries are more susceptible to cancer than the females regardless of the sex of the pituitary gland they carry, gives evidence that the sex of this gland does not play a role in this phenomenon. This concept is also supported by previous observations from this laboratory(4) showing that the continuous non-cycling ovarian stimulation in castrate males with transplanted ovaries as opposed the cyclic type in females does not depend on the sex of the pituitary gland *per se* but upon a sex difference residing in some other controlling structure.

In the light of the data presented it is difficult to decide as to where and how the sex difference in ovarian stimulation resides and operated. The fact that the "sex" of the hypophysis does not determine the sex pattern of activity of the ovaries indicates that there is a sexually differentiated structure somewhere else in the body which determines the sex pattern of gonadotrophic hypophyseal activity. This structure might be a part of the central nervous system, perhaps the anatomically contiguous hypothalamus. However at present no facts are available which would allow one to localize the site of the mechanism.

Summary. The incidence of mammary cancer and average cancer age have been determined in the following groups of AZ F1 hybrid mice: 1) sham ovariectomized non-breeding females; 2) ovariectomized females bearing their own ovaries transplanted; 3) hypophysectomized - castrate males bearing transplanted ovaries and female pituitaries; 4) hypophysectomized-castrate males bearing transplanted ovaries and male pituitaries. The results showed no significant differences in cancer incidence between any of the groups studied. However castrate males with transplanted ovaries developed cancer significantly earlier than did the females regardless of the sex of the pituitary gland they carried. Therefore it was tentatively concluded that the greater susceptibility to breast cancer observed in males is not dependent upon the sex of the hypophyseal gland *per se* but rather on a sex difference in some other structure controlling the hypophysis.

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Effect of Some Analgesic and Anti-Inflammatory Agents on Sodium Pentobarbital Anesthesia. (24065)

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It is commonly supposed that combinations of barbiturates with low-potency analgesics clinically show some type of "synergism" in relief of pain or production of increased hypnosis. In this communication we wish to report the effect of analgesics and anti-inflammatory drugs on brain barbiturate concentration under anesthesia, in order to gain insight into the mechanism of the increased hypnosis mentioned above. Duration of hypnosis was assessed concomitantly.

Methods. 1. *Prolongation of pentobarbital sleeping time.* Carworth male mice ranging in weight from 20-24 g were fasted for a period of 18 hours and distributed into 6 groups of 10 animals each. The compound under test was administered orally to 3 of these groups. One hour later, these groups received logarithmically spaced doses of sodium pentobarbital intraperitoneally. The same doses of sodium pentobarbital were administered to the remaining 3 groups of mice, previously untreated, which served as controls. Barbiturate-induced hypnosis was measured as a function of the duration of loss of the righting reflex (sometimes referred to as "sleeping time"). Relative potencies of pentobarbital with and without adjuvants were obtained by the method of Finney(1).

2. *Brain pentobarbital determinations.* Sodium pentobarbital levels in mouse brains were determined by the procedure of Brodie *et al.*(2) with some modifications: Carworth Farms male mice weighing 17-24 g were fasted overnight. Compounds tested were administered orally one hour prior to intraperitoneal administration of sodium pentobarbital (65 mg/kg). Two hours after sodium pentobarbital administration, the animals were killed by cervical dislocation and the brains removed immediately. Four mouse brains, having a total weight of approximately 1.7 g were homogenized in 4 ml distilled water. The homogenate was placed in a glass stoppered extraction tube to which was added 1.5 g sodium chloride, 1.0 ml buffer pH 5.5, and 60 ml petroleum ether containing 1.5% isoamyl alcohol. The petroleum ether-isoamyl alcohol solution was added in the cold room. The tubes were shaken in an Eberbach shaker (approximately 100 oscillations/min, 4 cm path), in a cold room at 4°C. At the end of 1 hour, a 40 ml aliquot of the petroleum ether phase was removed and shaken with 4 ml buffer pH 11 (4.25 ml 10 N NaOH + 200 ml Na_2HPO_4) for 3 minutes at room temperature. The petroleum ether phase was withdrawn and the aqueous phase transferred to a centrifuge tube. It was necessary to centrifuge in order to separate the aqueous from the petroleum ether phase completely. The aqueous phase was then read in Beckman DU Spectrophotometer at 240 mμ. Standards were prepared by taking 1 ml of various dilutions of a sodium pentobarbital solution and adding these to 5 ml buffer pH 11. Optical densities obtained with these dilutions were equivalent to the standard values obtained when sodium pentobarbital was carried through the extraction procedure. Sodium pentobarbital added to brain homogenate at concentrations of 10-100 μg showed a mean recovery of 93-99%. The brains of untreated mice were assayed simultaneously with those of mice receiving compounds but no sodium pentobarbital. In order to allow for day to day variations in response of the animals to sodium pentobarbital, controls receiving sodium pentobarbital alone were run with each experimental group. Brain blanks varied from 1.9-5.6 μg/g of brain.

Results. Significant prolongation of barbiturate hypnosis was produced by previous administration of aminopyrine or phenylbutazone at 25 mg/kg (Table I). Prednisone (25 mg/kg), morphine (1 and 25 mg/kg, or acetylsalicylic acid (25 or 250 mg/kg) which were also tested but not listed in Table I did

TABLE I. Effect of Aminopyrine and Phenylbutazone on Duration of Barbiturate Hypnosis.

Agents	Dose (mg/kg)	No. anesthetized*	Duration (min.)	Potency (90% confidence limits)
Na pentobarbital	65	10	162.4	1.
	48	10	75.6	
	35	8	13.7	
Idem + aminopyrine (25 mg/kg)	65	10	240.0	1.34 (1.21–1.49)
	48	10	153.0	$p < .001$
	35	7	108.0	
Na pentobarbital	65	10	106.0	1.
	48	10	79.6	
	35	7	9.0	
Idem + phenylbutazone (25 mg/kg)	65	10	232.6	1.28 (1.15–1.46)
	48	10	104.6	$p < .001$
	35	10	25.1	

* No. anesthetized from groups of 10 mice.

TABLE II. Effect of Several Agents on Brain Na Pentobarbital Concentration (μg/g Brain).

	Statistical analyses*					
	x		S.E.†		N	P
Compound (mg/kg)	Test	Control	Test	Control		
Phenylbutazone (25)	20.7	14.3	1.5	1.5	18	<0.01
Aspirin (250)	13.2	11.9	1.0	1.0	24	N.S.
Prednisone (25)	10.2	12.1	2.0	2.0	24	”
Morphine (25)	15.8	15.7	1.1	1.1	24	”
Aminopyrine (25)	29.7	17.7	1.4	1.4	30	$<<.001$

* x = mean; S.E. = stand. error; N = total No. of determinations divided equally among test and control groups; P values obtained by Student's "t" test; N.S. = not significant at $p = 0.05$.

† Day to day variation was seen in brain barbiturate levels, but were not significant statistically.

not elicit this effect.

The results summarized in Table II indicate that mice receiving sodium pentobarbital pretreated with phenylbutazone or aminopyrine show significantly increased brain barbiturate levels while those animals receiving prednisone, morphine or acetylsalicylic acid do not.

Discussion. One is tempted to speculate that those compounds producing prolongation of sleeping time and increased brain barbiturate levels may act by increasing permeability of the "blood-brain barrier," as apparently does physostigmine(3). However, it should also be considered that the phenylbutazone and aminopyrine effects may be due to inhibition of barbiturate metabolism as has been reported for many compounds(4). Our experiments throw no light on the mechanism of the effect which has been described. It would appear that the clinical impression that increased duration of hypnosis occurs with these combinations may be valid. It likewise appears that this effect is not related to anti-inflammatory action or analgesic potency.

Summary. The effect of acetylsalicylic acid, aminopyrine, morphine, phenylbutazone and prednisone on duration of hypnosis and brain barbiturate concentrations in pentobarbital anesthesia in mice has been determined. Among these agents, only aminopyrine and phenylbutazone significantly increased the brain barbiturate concentration and prolonged pentobarbital hypnosis. The other agents were without significant effect on these parameters.

We wish to acknowledge the help of Mr. Gordon Thomas, Schering Corp., Bloomfield, N. J. for the statistical analysis.

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Ribonuclease Inhibition of Mevalonic Acid Utilization.* (24066)

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Mevalonic acid (MVA) is a highly active biological precursor of the "isoprenoid" structure or of structures derived from this configuration. MVA is, therefore, readily converted to squalene or cholesterol in a number of systems. During the course of studies on factors influencing incorporation of MVA into cholesterol (CHL) or non-saponifiable material (NSF) by rat liver homogenates(1) it has been observed that the capacity for MVA utilization may be abolished by a predigestion of the system with crystalline ribonuclease (RNAse). The mechanism of this inactivation has not been fully elucidated but the technic for blocking biosynthesis at the MVA stage would appear to have sufficient implications to warrant detailed publication of the technic and initial findings at this time. A preliminary report, describing the utility of the procedure to demonstrate that mevaldic acid is convertible biologically into MVA has appeared(2).

Materials and methods. The experimental methods involved in the CHL biosynthesis studies have been described in detail(1). In brief, liver from young rats is homogenized with a "loose" homogenizer and incubated under oxygen with labeled precursors. The digest is then saponified and a NSF prepared which may be counted as such or treated with digitonin to form CHL digitonide which is similarly counted. In the experiments involving predigestion with RNAse or other enzyme prior to the addition of C^{14}-MVA or C^{14}-acetate, the flasks receiving the precursor were given a second aeration with oxygen at the time of addition. All incubations were carried out for 4½ hours from the start. RNAse used in most of the experiments was 3X crystallized material from Worthington Biochemical Corp., Freehold, N.J. This material is described as prepared by the McDonald modification(3) of the Kunitz procedure to render it free of protease activity. In addition, absence of protease activity was confirmed in these laboratories by the use of a highly sensitive method(4[†]). Similar results were obtained with crystalline RNAse from Nutritional Biochem. Corp., Cleveland, Ohio. Where microbiological assays for MVA were carried out samples were prepared by removing 0.1 ml aliquots from the incubation mixtures at the conclusion of the experiments, diluting to 10 ml and heating at 100° for 10 min. Microbiological determinations of MVA were carried out with *Lactobacillus acidophilus* 4963 according to the method of Skeggs *et al.*(5). All values for MVA are expressed in terms of the DL dibenzylethylenediammonium (DBED) salt used as a standard in the microbiological assays even though the C^{14}-MVA actually used in the enzyme experiments was added as the sodium salt prepared by extraction of the DBED by ether from alkaline solution. Two preparations of 2-C^{14}-MVA were used[‡], one (A) had an activity of 15,500 cpm/mg, the other (B) had an activity of 36,000 cpm/mg. The C^{14}-acetate used had an activity of 1,250,000 cpm/mg.

Results. Preliminary experiments showed that when MVA and RNAse are added at the same time to an active liver homogenate the RNAse is without effect on the activity of the CHL isolated. In subsequent experiments the effect of various enzymes has been tested by allowing the enzymes to act for varying periods of time before the labeled substrates are added. Under the conditions described (Table I) utilization of MVA as determined by counts found in the NSF or CHL or by the amount of MVA remaining by microbiological assay is only slightly impaired by a

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[*] This investigation was supported by grants from Nat. Science Fn. and Nat. Inst. of Health.

[†] We are indebted to Doctors G. P. Hess and E. Wainfan for determinations of proteolytic activity.

[‡] 2-C^{14}-MVA was kindly supplied by Drs. J. M. Sprague and C. S. Miller, Merck Sharp and Dohme Laboratories, West Point, Pa.

TABLE I. Biosynthesis of Non-Saponifiable Material and Cholesterol Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with 5 mg of Ribonuclease for Varying Time Intervals.

Exp.	MVA addition, min.	NSF, cpm/mg	CHL, cpm/mg	MVA found, mg
1	0		1435	
	0		1728 (0)	
	15		1805	
	15		785 (56)	
	30		1422	
	30		31 (98)	
2	0		2042	
	0		2000 (2)	
	15		2582	
	15		928 (64)	
	30		1960	
	30		8 (100)	
	60		745	
	60		5 (100)	
3	0		1915	
	0		1865 (2)	
	15		1750	
	15		986 (54)	
	30		1985	
	30		0 (100)	
	60		1429	
	60		0 (100)	
4	0	440	1172	.0
	0	481 (0)	971 (17)	.0 (0)
	15	365	1142	.0
	15	270 (26)	536 (53)	.3 (30)
	30	358	725	.0
	30	1 (100)	2 (100)	1.0 (100)
	60	4	6	1.0
	60	0	4	1.0

1 mg 2-C^{14}-MVA (A) added at the times indicated followed by a second flushing with oxygen.

Figures in parentheses indicate % inhibition of either biosynthesis or utilization of precursor.

preliminary digestion for periods up to 30 min. Some liver preparations even retained essentially unchanged their capacity for NSF and CHL biosynthesis after incubation for a period of 60 min. On the other hand, complete inactivation of MVA utilization is encountered after a preliminary digestion for 30 min with 5 mg of RNAse. One-half inactivation is obtained with about 100-200 γ per flask (Table II). Incorporation of MVA into cholesterol is not impaired by a similar preliminary digestion with DNAse. Other experiments have shown elastase and transacetylase to be inactive. Incorporation of acetate into NSF or CHL also does not occur in RNAse-treated liver (Table III). In all instances where both microbiological assays for utilized MVA and determinations of MVA incorporation into NSF or CHL have been carried out it was found that incorporation is correlated directly with MVA disappearance. Where RNAse inhibition was complete essentially quantitative recovery of unused MVA is found. MVA is found at the conclusion of an experiment only when the homogenate contained added MVA *and* added RNAse. (Table IV). Presumably in the absence of added MVA there is not enough "endogenous" MVA to be determined. Similarly in the absence of added RNAse the amount of MVA added does not exceed the capacity of the tissue for biosynthesis and the added factor is readily utilized.

Discussion. Bucher and McGarrahan previously concluded(6) that incorporation of acetate into CHL by fractionated rat liver homogenates is not influenced by small amounts of RNAse added to incubation mixtures at the same time as labeled precursor. As pointed out earlier in this paper, RNAse is without significant effect unless given a "head start" before precursor is added.

Some comment is in order concerning the amount of RNAse required to produce the effects reported here. Comparison of the RNAse required to inactivate certain protein-synthesizing enzyme systems with that required to inactivate the CHL-synthesizing system indicates that the requirement in the present system is quite high. The nature of the substrate involved in the present experi-

TABLE II. Biosynthesis of Non-Saponifiable Material and Cholesterol Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with Various Amounts of Ribonuclease.

RNAse, mg	NSF, cpm/mg	CHL, cpm/mg	MVA found, mg
0	1084 (0)	2557 (0)	.0 (0)
.5	298 (72)	1040 (60)	.31 (62)
1.0	192 (83)	620 (76)	.45 (90)
2.0	130 (88)	385 (86)	.45 (90)
5.0	36 (97)	94 (97)	.63 (126)

.5 mg 2-C^{14}-MVA (B) added after 30 min. from start of experiment followed by a second flushing with oxygen.

Figures in parentheses indicate % inhibition of either biosynthesis or utilization of precursor.

ments, however, is unknown and it is entirely conceivable that the degradation of an "atypical RNA" or other RNAse-sensitive compound may require somewhat more enzyme than required for degradation of a more "typical" RNA.

In searching for an explanation for the phenomenon here described, the possibility has been considered that the inactivation obtained with RNAse may result from interference with the structure of tissue particles and consequent disorganization of the enzyme systems that they contain. Although a number of studies have emphasized the significance of the microsomes in CHL biosynthesis the microsomes do not appear essential for squalene biosynthesis(7,8,9). It may be pointed out in addition that pre-digestion with RNAse has been reported to be without effect on the glucose-6-phosphatase, esterase, triacetic acid lactonase, adenosine - 5′ - phosphatase, acid phosphatase, adenosine triphosphatase, and succinoxidase activity of isolated rat liver microsomes(10). Mitochondria do not appear to be essential for either squalene or CHL biosynthesis(7.8.9).

The possibility was considered that the effects observed with RNAse are due to inhibitory compounds formed from RNA by the enzyme. In a separate experiment an amount of commercial RNA equivalent to the RNA calculated to be present in the liver homogenate used was digested separately with RNAse and then added to homogenate along with MVA. No inhibitory effect was observed.

Some consideration should be given to a mechanism whereby RNAse functions only indirectly in inhibition of NSF and CHL biosynthesis, for example, by interfering with various supporting energy or reductive mechanisms that appear to be essential for conversion of MVA to NSF or CHL.

TABLE III. Biosynthesis of Non-Saponifiable Material and Cholesterol from Mevalonate or Acetate Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with Ribonuclease.

Exp.	MVA	Acetate	RNAse	NSF	CHL	MVA found,
	mg			cpm/mg		mg
1	.5			351	489	0
	.5		5	118	95	.47
2		1.0		2857	7315	0
		1.0	5	16	7	0

.5 mg 2-C^{14}-MVA (B) or 1.0 mg C^{14}-acetate was added 30 min. from start of each exp. followed by a second flushing with oxygen.

TABLE IV. Biosynthesis of Non-Saponifiable Material and Cholesterol Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with Ribonuclease.

Exp.	MVA	RNAse	NSF	CHL	MVA found,
	mg		cpm/mg		mg
1	.5		259	1380	0
	.5	5	23	120	.64
2					0
		5			0
	.5		369	930	0
	.5	5	135	61	.50

2-C^{14}-MVA (B) where used was added 30 min. from start of each exp. followed by a second flushing with oxygen.

Obviously further work is required to provide a satisfactory explanation for the experimental data obtained. Pending further elucidation of the mechanism of RNAse inactivation, the technic described stands as a convenient and useful procedure for blocking isoprenoid biosynthesis at the MVA stage.

Summary. Utilization of mevalonic acid as determined by incorporation of the 2-C^{14}-labeled compound into non-saponifiable material or into cholesterol or by disappearance of microbiological activity attributable to mevalonic acid in rat liver homogenates was found to be abolished by a preliminary digestion with ribonuclease.

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Preservation of Human Epithelial-Like and Fibroblast-Like Cell Strains at Low Temperatures.*† (24067)

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The development of numerous strains of mammalian cells has created many problems with regard to their maintenance. For example human epithelial-like (Ep-L) cell strains undergo biologic changes both during their development into "stable" strains and while being maintained under conditions of rapid growth and frequent subculture(1). In addition, human fibroblast-like (Fb-L) cell strains may be difficult to maintain as continuous cell lines(1). Scherer and Hoogasian(2) studied various methods of preserving cell strains at low temperatures, and observed that either fast or slow freezing in 20-30% glycerol medium, storage at –70°C, and rapid thawing, were optimal conditions for preserving HeLa cells. Swim *et al.*(3), have found that survival of their cell strains depended on methods of freezing and glycerol concentration, as well as on methods of cultivation prior to freezing and on sources of their strains. Experience in our laboratories has shown that although rapid freezing in 20% glycerol medium could be used for preservation of various Detroit Ep-L strains for as long as one year, the technic was not a satisfactory solution to the problem because cells were damaged during the freezing and their recovery was unpredictable. Moreover, by that procedure it was not possible to recover human fibroblast-like strains at all.

In a recent review of the mechanics of freezing(4), it was pointed out that cell damage might be minimized if non-toxic concentrations of a water binder are allowed to pass through cell membranes over temperature ranges where intracellular crystallization and dehydration denaturation usually occur. This principle was applied in the present study. This communication describes a procedure of slow freezing in 5% glycerol medium, storage at –70°C, and rapid thawing, as an efficient and practical means of preserving both epithelial-like and fibroblast-like human cell strains.

Procedure. Establishment and characterization of 17 epithelial-like (Ep-L) human cell strains employed, have been described (1,5,6). The fibroblast-like (Fb-L) human cell strains were derived from a teratoma of the testes (Detroit-504 Fb-L) and from kidney (Detroit-506 Fb-L) by methods previously described(7). The cells of various strains were cultivated in 3-ounce prescription bottles with Eagle's basal medium(8) supplemented with 16% human serum. Cellular monolayers occurring in 7-day-old cultures were treated with 0.25% trypsin in calcium-free Hanks' balanced salt solution for 10 minutes at room temperature. The resulting cell suspensions were centrifuged and resuspended in a medium consisting of basal medium 75%, human serum 20%, and glycerol 5%. Following cell counts made with a hemacytometer, cell suspensions were appropriately diluted (and cells recounted) so as to contain approximately 5 x 10^5 cells per ml. One ml aliquots were transferred to ampules which were then either glass-sealed or rubber-stoppered. In earlier experiments, slow freezing was accomplished by immersing the ampules in an ice-alcohol bath at approximately –4°C and then slowly lowering the temperature to –15°C over a period of 30 minutes. The ampules were then placed in a freezer maintained at –25°C until their contents were solidly frozen, then transferred to a dry-ice chest maintained at –70°C for permanent storage. This will be referred to as procedure "A". More recently, we have used a mechanical slow-freeze apparatus‡ with

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† Presented in part at Annual Meeting of Tissue Culture Assn., Philadelphia, April 10, 1958.

‡ Manufactured by Canal Industrial Corp., Bethesda, Md.

TABLE I. Preservation and Recovery of Human Cell Strains by Slow Freezing in 5% Glycerol-Medium, Storage at –70°C and Rapid Thawing.

				Cell counts × 10^5			
						Harvested from	
Cell strain (Detroit)	Cell type	Slow-freeze procedure	Months stored (–70°C)	Frozen	Thawed	7-day culture of thawed cells	Subcultures (inoculum 3×10^5, cultured 7 days)
6	Ep-L	A	6	5.1	4.9	36.8	82.5
32	"	A	6	5.4	4.5	20.5	60.2
34	"	A	6	4.7	4.7	27.0	92.0
52	"	A	6	3.6	2.9	21.0	46.3
56A	"	A	6	4.0	4.2	16.0	48.6
98	"	A	6	3.4	2.8	23.9	35.6
196 Clone V	"	A	2	5.5	4.5	72.0	
" "	"	B	2	5.5	4.8	86.0	
" III	"	B	2	5.4	5.1		
B16	"	A	6	5.5	4.4	25.2	74.0
" I	"	B	2	5.4	4.7		
" II	"	B	2	5.0	5.6		
B17	"	A	6	5.1	4.7	46.0	80.6
B78	"	A	6	4.3	4.5	21.3	71.9
" I	"	A	1	4.7	4.5	22.8	106.0
" I	"	B	1	4.7	5.0	39.6	103.0
" II	"	A	1	5.3	3.0		
" II	"	B	1	5.3	3.6		
173B	"	A	6	4.8	4.8	68.0	109.0
501	"	A	6	4.9	5.5	19.8	50.2
504	Fb-L	A	1	4.7	3.5	6.7	21.0
506	"	A	1	5.1	3.5	8.0	
"	"	B	1	5.1	3.8		

which it was possible to lower the temperature at a controlled rate of one degree per minute until it reached –25°C. At this point, the temperature was quickly lowered to –70°C, at which level the cells were permanently stored. The controlled rate method will be referred to as procedure "B". Cell suspensions were thawed within 30 seconds by placing ampules under running water at 37°C. The entire contents of an ampule were diluted to 8 ml with growth medium (basal medium + 16% human serum, pH 7.2), and after determination of the number of thawed cells present, were cultured according to the procedure described above. Replicate bottle cultures of thawed cells were prepared to determine their growth curves by cell counts.

Results. In Table I are listed examples of each of 17 epithelial-like cell strains frozen by procedures A or B, and stored at –70°C. With the single exception of Detroit-B78 Clone II, a comparison of the number of Ep-L cells frozen to the number seen immediately after thawing revealed that 80% to 100% appeared undamaged (intact) as viewed in the counting chamber. Each of the thawed cell strains was then cultured as described (the medium changed on 3rd and 6th day), and resultant cell yield was determined at end of 7-day incubation periods. In most instances these cell yields were lower than those usually obtained from weekly serial passage. This would suggest that not all the thawed cells counted were actually capable of reproduction. From the harvested cells uniform cell inocula (3 x 10^5 cells) were prepared which allowed comparisons of growth rates of recovered strains to those regularly maintained by routine subculture every 7 days. The cell yields obtained from each recovered strain after one subculture were of the same order of magnitude as unfrozen strains. No changes in the appearances of cell monolayers or individual cell morphology could be detected. Also, thawed cells of clonal strains were recovered in numbers equivalent to those recovered in the parent strain cultures and when cultured, gave yields equivalent to unfrozen strains (Table

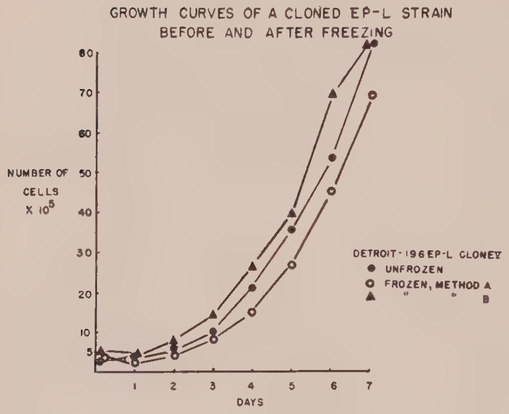

FIG. 1. Growth curves of Detroit-196 Ep-L Clone V cell strain. Curves depict rate of multiplication of unfrozen cells compared to the thawed cells which had been frozen according to procedures A and B.

I). Of such strains studied, only one, Detroit-B78, derived from normal peripheral blood (6), yielded 2 clonal strains that were affected differently when frozen simultaneously either by procedure A or B. The number of cells recovered from the original cell line and one of the clonal lines were similar. Detroit-B78 Clone II, however, yielded fewer cells after thawing.

The 2 fibroblast-like strains, Detroit-504 Fb-L and -506 Fb-L, were subjected to the freezing procedures. Although the number of cells recovered immediately after thawing was somewhat lower than from Ep-L strains (65-75% as compared to 80-100%), the thawed cells could easily be cultured with no change in morphology or growth rate. Cell yields of established Detroit Fb-L strains have always been characteristically lower than Ep-L strains. It is noteworthy that 3 months after its isolation as a stable Fb-L strain, growth rate of Detroit-504 Fb-L diminished and eventually the strain was lost. Cells frozen before this event, however, have been consistently recovered from the frozen state, as illustrated by the example in Table I. Moreover, the recovered strains have been continuously maintained by serial subculture in several laboratories in addition to our own. Because of the importance of fibroblast-like strains for certain virologic problems(7,9), the ability to preserve them in this fashion is of considerable practical significance.

Fig. 1 illustrates the growth curve of an unfrozen clonal strain of Ep-L cells as compared to the same strain frozen by method A and method B, and stored for 2 months at –70°C. The similarities of the curves indicate that most, if not all, of the thawed cells retained their ability to multiply.

Discussion. These results indicate that the controlled rate slow freezing procedures are a practical and efficient means for preserving various kinds of human cell strains. Moreover, a comparison of rapid and slow methods of freezing expose differences between human fibroblast-like (Fb-L) and epithelial-like (Ep-L) cell strains. With rapid freezing technics, Fb-L cells cannot be recovered, but with the slow freeze methods described, the Fb-L cells can be recovered successfully. The practical significance of this is that it has been shown that human Fb-L cell strains are capable of supporting the propagation of viruses not able to multiply readily in Ep-L cell strains(7,9), yet human Fb-L strains are difficult to maintain indefinitely by serial subculture. The fact that the fibroblast-like form of human cell strains cultured by present methods usually represents a temporary stage in the development of epithelial-like lines(1), and the relative rarity of long term human Fb-L cell lines, makes it important to utilize methods which will insure a supply of human cell strains in the Fb-L state. We feel that the freezing procedures described herein satisfy these requirements. A more obvious application of these procedures is the circumvention of difficulties involved in continuous maintenance of large collections of cell strains such as cost, contamination, changes in virus susceptibility, and cell variation.

Summary. Studies of cell preservation by slow freezing in 5% glycerol medium, storage at –70°C, and rapid thawing were carried out on 17 epithelial-like and 2 fibroblast-like human cell strains (Detroit). Following this procedure, each of the strains could be quickly recovered as actively proliferating cell strains with no changes in growth rate or strain characteristics.

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An Orally Active Compound with Anti-Fertility Effects in Rats.* (24068)

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Inhibition of fertility in rats can be achieved by interfering with normal mechanisms at any one of several vulnerable points in the reproductive process. These include impairment of gametogenesis by compounds having direct action on germ cells(1,2), prevention of ovulation by administering steroids which suppress pituitary production of gonadotrophins(3), withdrawal of progestational support of the endometrium(4), or destruction of developing embryos with anti-metabolites(5). The use of estrogens to terminate pregnancy in rats was reported 3 decades ago by Smith(6). Subsequently, numerous investigations established that high dosages of estrogen cause tubal blockage when administered immediately after fertilization, while later treatment interferes with maintenance of a progestational endometrium(7,8). A type of activity completely different from any of those mentioned above appears to be characteristic of a particular triphenyl ethanol derivative, 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol.† When female rats are treated for a brief post-copulatory period with an adequate dose of this compound, implantation never occurs. These animals enter a period of pseudopregnancy, characteristic of rats following sterile mating, and upon resumption of estrous cycles, are completely capable of bearing normal litters(9). This report presents experiments leading to these observations. We have attempted to establish the phase of the reproductive process influenced by MER-25, dosage requirements for the compound's anti-fertility effects, and site of its activity.‡

Methods. Fertile female rats of Sprague-Dawley strain were placed with proven male breeders during early evening and vaginal smears were examined for spermatozoa the following morning. The day on which sperm were found in the vaginal smear is considered day 1 of pregnancy. On this basis, days 1-3 and part of day 4 comprise the period of oviducal passage. The remainder of day 4 and day 5 follow tubal exit when blastocysts are free in the uterus and days 6, 7, 8 are the period of implantation. Formulating dosage schedules in relation to these reproductive events, MER-25 was administered by stomach tube on a body weight basis. The effectiveness of systemic administration has been reported(10). Concentrations were established so that the animals uniformly ingested 0.1 ml of olive oil solution/100 g body weight. Body weights and vaginal smears were recorded daily. Eight to 12 days post-coitus, the animals were laparotomized and both uterine horns

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* Assistance for this work was provided by Grant from NIH, USPHS.

† This compound referred to as MER-25, was kindly supplied by Wm. S. Merrell Co., Cincinnati, O.

‡ We are indebted to C. R. Thompson, Director of Pharmacology, Cutter Laboratories, for his suggestion of potential anti-fertility activity of this compound.

TABLE I. 4-Day Treatment with MER-25, 25 mg per kg Daily Oral Administration.

Treatment days post-coitus	No. of animals	No. of pregnancies	Avg duration pseudo pregnancy (days)
1– 4	20	0	15
2– 5	8	0	15
3– 6	8	0	14
4– 7	8	0	15
			Avg litter size
5– 8	8	8	9
1– 4 (control)	15	15	10
	Continuous treatment		
5–21	4	4	9

were examined for implantation sites. Number of animals employed in each experimental group is indicated in the tables. For histological study of developmental events occurring prior to tubal exit, oviducts were recovered from treated and untreated animals, 1, 2, 3 and 4 days after mating, fixed in Bouin's or Zenker's fluid and serially sectioned. To determine if uteri of treated animals retain capacity to form decidua, 3 mated females were treated with MER-25 during the first 4 days post-coitus and then subjected to artificial uterine irritation. On day 11, these animals were sacrificed and the irritated sites sectioned and examined for decidual reactions.

Results. Timed - administration experiments. When treatment with 25 mg/kg body weight was initiated on day of mating and continued throughout normal period of oviducal passage of ova (days 1-4), implantation never occurred (Table I). Laparotomy on day 12 revealed absence of nidation sites. Animals treated on days 1-4 regularly entered a period of pseudopregnancy of 15 days average duration, then resumed normal estrous cycles. Subsequent matings with the same breeding males proved fertile. Gestation was likewise prevented in all instances when treatment included any part of the oviducal period and was continued for 4 days (days 2-5, 3-6, 4-7). When treatment of similar length was initiated after tubal exit of the ova (days 5-8), not a single pregnancy was terminated and the fetuses developed normally. Litter sizes in these cases were not significantly different from controls. It has been possible to administer the compound continuously following period of oviducal passage without impeding gestation, causing fetal resorption, or reducing litter size (Table I).

Daily administrations of 25 mg/kg of the compound for 1, 2, or 3 days, including portions of the period of oviduct passage, drastically reduced fertility, although occasional pregnancies did result (Table II). These occurred mainly when the abbreviated treatment period was initiated immediately after copulation. When begun later in the oviducal period and extended into the period of the free blastocyst, the 3-day or 2-day treatment was essentially 100% effective in preventing implantations. Single, daily ingestions of 25 mg/kg body weight, regardless of day of administration, failed frequently to provide absolute protection against implantation and gestation. That this may merely reflect a dosage phenomenon is indicated by the results of increasing treatment to 2 or 3 administrations of 25 mg/kg during the first post-copulatory day. The b.i.d. treatment significantly reduced the number of successful pregnancies to only 1 out of 8, while the t.i.d. regimen proved 100% effective in 12 cases. For the purposes of a 4-day treatment period, however, the dosage of 25 mg/kg is well above

TABLE II. 3-, 2- or 1-Day Treatment with MER-25, 25 mg/kg Daily Oral Administration.

Treatment days post-coitus	No. of animals	No. of pregnancies	Litter size
1–3	10	3	1, 1, 2
1–2	8	3	7, 3, 1
1	8	6	11, 7, 5, 3, 2, 2
1 (2 × 25 mg/kg)	8	1	6
1 (3 × 25 ”)	12	0	
2–4	10	1	11
2–3	8	0	
2	8	3	8, 8, 7
3–5	10	0	
3–4	8	1	6
3	8	3	11, 8, 5
4–6	10	0	
4–5	8	0	
4	8	3	9, 9, —
5–7	5	5	9 (avg)
5–6	8	8	9 ”
7–8	4	4	10 ”

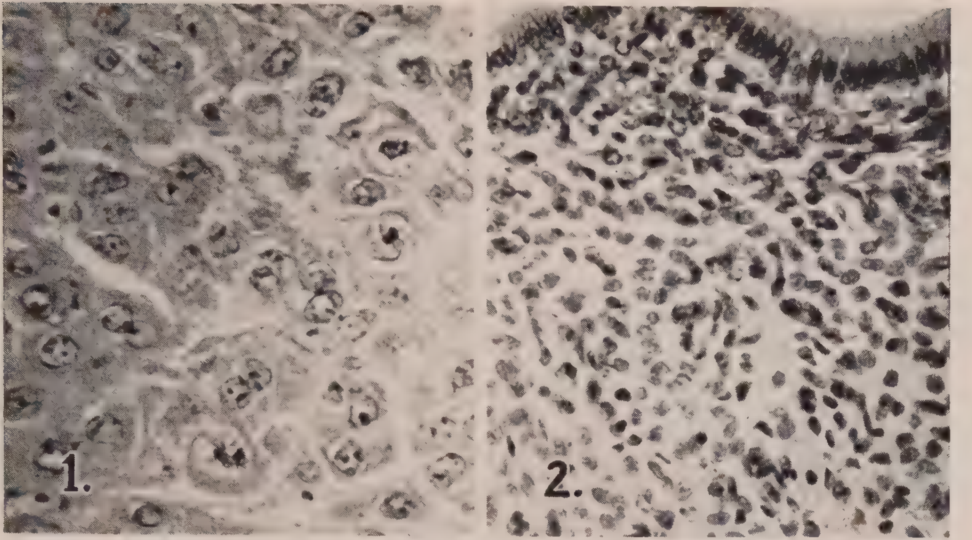


FIG. 1. Cross-section of artificially stimulated region of MER-25 treated rat uterus. Note decidual cells and stromal edema (×340).

FIG. 2. Cross-section of contralateral, unstimulated uterine horn at same magnification as Fig. 1. Histologic appearance is typical of pseudopregnancy.

the minimal effective dose. Absolute prevention of pregnancies was achieved with 20 mg/kg and 15 mg/kg. The dose of 10 mg/kg was effective in most instances, and when this low dosage failed to prevent pregnancy completely, there was a severe reduction in litter size (Table III).

Artificial stimulation of endometrium. Uteri of animals treated daily with 25 mg/kg MER-25 are capable of forming decidual reactions at time of normal implantation. Uterine sites, irritated by needle-scraping, develop typical deciduomata (Fig. 1) within one week after artificial stimulation. Enlarged decidual cells exhibit a high incidence of mitoses; stromal edema and vascularization is pronounced. There were no implantation sites in the undisturbed, contralateral uterine horn of these mated, then treated animals. The histologic appearance is that of normal pseudopregnancy (Fig. 2).

TABLE III. MER-25 Treatment, Days 1-4 Post-Coitus Oral Administration.

Daily dose, mg/kg	No. of animals	No. pregnancies	Litter size
25	20	0	
20	12	0	
15	12	0	
10	12	2	1, 3

Developmental events within oviducts. Gross morphology, average tubal diameters, and histologic characteristics of oviducts from treated animals do not differ from controls. Within 24 hours after mating, sections through newly fertilized eggs of treated animals show no abnormalities. Extrusion of 2nd polar body and fusion of pronuclei appear

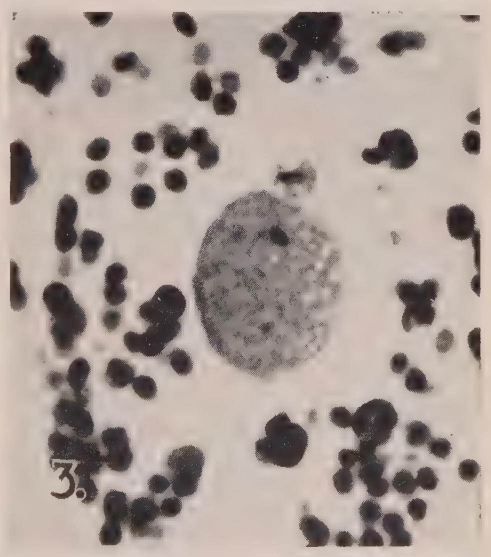
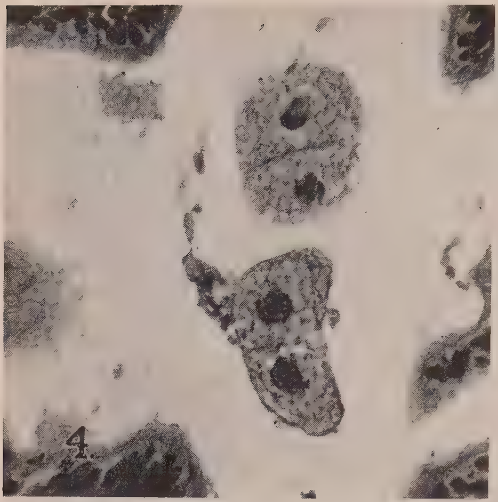
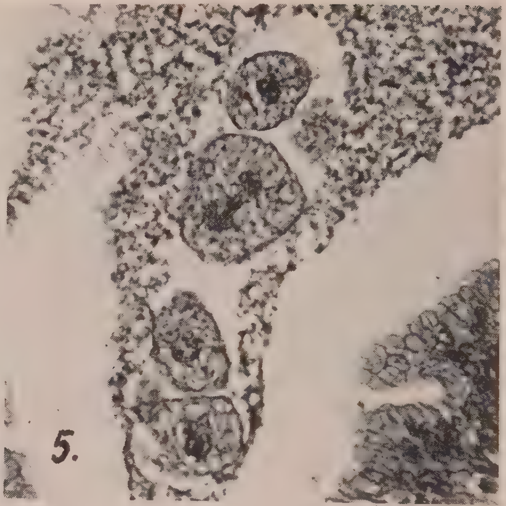
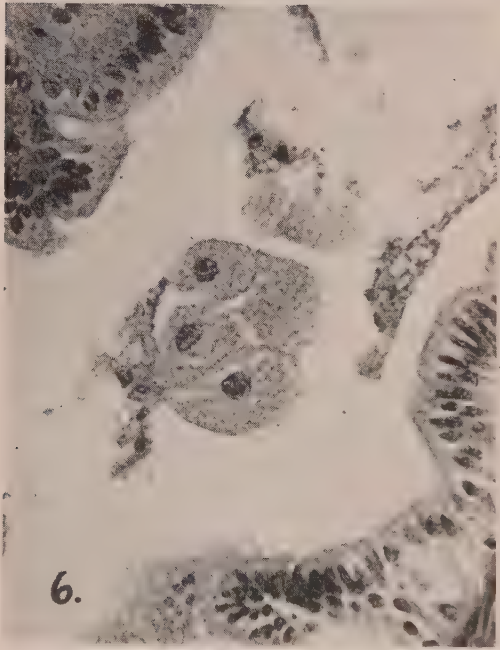


FIG. 3. Tubal ovum within 24 hr post-coitus. Animal was treated with MER-25 12 hr prior to sacrifice. Note normal fusion of pronuclei and extruded 2nd polar body (×530).







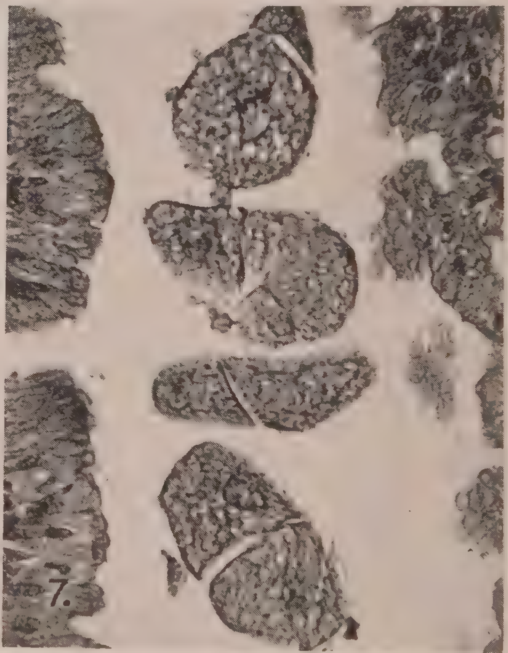


FIG. 4. Section through tubal ova of control animal sacrificed between 24 and 48 hr post-coitus. Each of these zygotes had reached the 4-cell stage (×340).

FIG. 5. Section through tubal ova of MER-25 treated animal sacrificed between 24 and 48 hr post-coitus. Blastomeres are of irregular size. Note arrested spindle apparatus (×340).

FIG. 6. 8-cell morula sectioned within the oviduct of a control animal sacrificed between 48 and 72 hr post-coitus (×340).

FIG. 7. Zygotic fragments found in oviduct of MER-25 treated animal sacrificed between 48 and 72 hr post-coitus. Note abnormal and unequal cleavages and beginning of fragmentation (×340).

to occur in normal fashion (Fig. 3). The beginning of first cleavage can frequently be observed. Between 24 and 48 hours most normally developing ova are in the 2-cell stage or have reached the 4-cell stage (Fig. 4). In treated animals sacrificed between 24 and 48 hours, more frequently than not, there are evidences of abnormal divisions with blastomeres of irregular size and arrested spindle apparatus (Fig. 5). Between 48 and 72 hours, the normal zygote has reached the 8- or 12-cell stage with daughter blastomeres of equal size (Fig. 6). Treated animals by this time show an apparent degradation of developing ova. Irregular and unequal divisions result in bizarre aggregates of blastomeres and many of the clusters start to fragment (Fig. 7). Pycnotic nuclear material is frequently seen. After the third day, between 72 and 96 hours post-coitus, neither developing eggs nor identifiable remnants of eggs could be found in oviducts of animals treated daily with MER-25. Normal animals sacrificed at the same time may be expected to show well-formed morulae approaching the utero-tubal junction.

Discussion. The period of effectiveness for achieving anti-fertility activity with MER-25 corresponds to the time necessary for passage of fertilized ova through the oviduct and the preimplantation phase of zygotic development *in utero*. Inasmuch as treatment was initiated many hours after mating, fusion of gametes and all phases of the reproductive process leading to this event could not be involved in the observed inhibition of fertility. We cannot rule out, however, the possibility that the compound may also act at a prior vulnerable point in the sequence of reproductive events; this warrants further investigation. It seems well-established, on the other hand, that the influence of MER-25 is exerted prior to and exclusive of the process of implantation.

Throughout these experiments, when length of treatment or dosage factors were not optimal and pregnancies resulted, there was frequently a reduction in litter-size. Ovaries of these animals contained normal numbers of functional corpora lutea and no fresh resorption sites were noted in the uterus. This indicates that a usual complement of ova were released at ovulation and that the reduced litter-size did not result from fetal resorption. It would appear, therefore, that the defect arises in the developing eggs themselves, or in the capacity of the uterus to accept blastocysts for implantation. However, the fact that some blastocysts do implant indicates that the uterus is capable of supporting nidations. This is confirmed by development of deciduomata following artificial stimulation of endometrium of treated animals. In addition, this reaction proves that the compound does not interfere with post-copulatory maintenance of luteal body function, for the decidual reaction could not occur in its absence. That the corpora lutea are functional is indicated also by vaginal smear records of successfully treated animals. Each of the 142 animals treated adequately for prevention of pregnancy entered a characteristic period of pseudopregnancy, a phenomenon that requires maintenance of functional corpora lutea.

It would appear, therefore, that by the time of implantation, there was only a partial survival of ova, below the number released at ovulation and subsequently fertilized. Those blastocysts that did implant developed normally and well-formed fetuses were delivered at term. These offspring showed no signs of anomalies whatsoever and were reproductively normal upon reaching sexual maturity. Fetuses developed normally also in those cases treated continuously following tubal exit (days 5-21), but normal parturition was prevented by MER-25 administration. The mechanism of this interference with parturition is not yet understood and is presently under investigation. It is likely that the effect is on the maternal endocrine balance and is related to estrogen antagonism by the compound. By virtue of extensive pharmacologic testing, Thompson and Lerner(11,12) have established that MER-25 inhibits the peripheral response of various end-organs to both exogenous and endogenous estrogenic stimulation. On the basis of the present observations, the compound's anti-fertility activity cannot be clearly linked with its known anti-estrogenic activity. This mode of action, however, must be considered as a distinct possibility.

It may be concluded that the anti-fertility effect of MER-25 results from annihilation of the zygotes as they descend the tubes or while they are free blastocysts in the lumen of the uterus. The mechanism of this cytotoxic effect remains to be established. Conceivably, the compound may act not on the eggs themselves, but on the maternal duct system in a manner so as to remove necessary support for normal zygotic development.

Summary. When MER-25 is administered orally to female rats on day of mating and continued throughout the normal period of oviducal passage of ova, implantation does not occur. Restricting treatment period to only one day post-coitus can also be effective in preventing nidation. Treated females enter a post-copulatory period of pseudo-pregnancy, then resume normal estrous cycles. The antifertility effects of MER-25 appear to be exerted prior to exit of ova from the oviducts. The compound does not act by preventing decidua development, nor by interfering with post-copulatory maintenance of luteal body function. Histologic evidence indicates that ova of females treated with MER-25 undergo degeneration after extrusion of 2nd polar body.

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Serum Cholesterol in Man and the Unsaponifiable Fraction of Corn Oil in the Diet.[*][†] (24069)

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A decrease in serum cholesterol level has been found when corn oil is used in the diet, in substantial amounts in man(1-6) and in the cholesterol-fed chick(7). It has been suggested that much of this effect is due to something besides the fatty acid glycerides in corn oil(8). For these reasons we excluded comparisons involving corn oil in our recent analysis of a large series of fat-feeding experiments(9,10). This analysis indicated that with many fats (butterfat, olive oil, cottonseed oil, sunflower seed oil, sardine oil and mixed fats of ordinary foods), a change in fat content of the human diet results in a change in serum cholesterol level which is predictable from a regression equation, cholesterol rising with increasing saturated and falling with increasing poly-unsaturated fats in the diet: Δ Chol. $= 2.74\ (S_2 - S_1) - 1.31\ (P_2 - P_1)$, (Equation 1) where S and P are percentages of total calories provided by glycerides of saturated and of poly-unsaturated fatty acids, respectively, and the subscripts refer to first and second diets. When data from dietary changes involving corn oil were tested with this equation, the results tended to show that corn oil produced slightly lower serum cholesterol values than predicted. This would conform to the suggestion that corn oil may contain a special cholesterol-lowering substance not accounted for as fatty acid glyceride, presumably in the unsaponifiable frac-

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tion. Accordingly, it was decided to examine this question further. In the first place, a more critical analysis was made of data from experiments reported previously. Three switchback dietary comparisons in which any time trends are automatically cancelled out were selected from Exp. H and J(11) and are separately analyzed below. Secondly, a new experiment (Exp. N) with this switchback arrangement was performed comparing safflower and corn oils. Finally, a similar experiment, Exp. O, was performed to test the effect of a concentrate of unsaponifiable matter in corn oil. All of these results are reported below.

Methods. Physically healthy schizophrenic male subjects were selected and controlled as in earlier experiments(11). Their ages were from 39 to 66 yrs. and relative body weights from 72 to 119% of standard for their age and height(12). Out of 98 subjects only 5 suffered acute illnesses or had body weight changes of 2 kg in a dietary period or 3 kg in an entire experiment. The data of these 5 men were excluded. A basic diet low in fat (about 40 g per day), as previously described (11), was devised so that calorie equilibrium would be achieved when 100 g of experimental fat were added to the daily ration by incorporating it in the basic diet just before serving by adding it to the cooked hot dishes. Plate rejections were recorded and the diet was adjusted as needed to keep body weight constant as in previous experiments(11). The experimental fats used in the 3 comparisons from Exp. H and J have been reported elsewhere(10,11). In Exp. N commercial samples of corn oil (Mazola) and safflower oil (Pacific Vegetable Oil Co.) were used. Their fatty acid composition was: corn oil—15, 26 and 59% saturated, mono-ene and polyene, respectively; safflower oil—12, 10 and 78% of these fatty acids. In Exp. O the plan was to measure the effect on serum cholesterol of addition to and removal from the diet of the unsaponifiable fraction of corn oil in the presence of a mixture of cottonseed and safflower oil (100 g daily) approximating corn oil in proportions of saturated and linoleic acids. Compositions of oils and the final mixture (imitation corn oil) are given in Table I.

TABLE I. Distribution of Glycerides of Saturated (S), Mono-ene (M) and Polyunsaturated (P) Fatty Acids in the Oils Used in Experiment O.

Oil	Amount in g Total	S	M	P
Cottonseed	56	13.4	12.9	29.7
Safflower	44	5.3	4.4	34.3
Mixture fed*	100	18.7	17.3	64.0

* Imitation corn oil.

Unsaponifiable fraction of corn oil. The unsaponifiable fraction in Exp. "O" was prepared from corn oil having an unsaponifiable content of 1.6%. Eighteen gallons of corn oil were saponified by refluxing one hour with 5.5 times its weight of methanol and 3 times the theoretical amount of NaOH. The pH was then brought to 9-9.5 with HCl, and the mixture was slightly cooled and dried. The dry flakes were washed in 300 gallons of acetone, the mixture being agitated mildly and extracted overnight at room temperature. Extraction was repeated twice more, the extracts were concentrated to about 8 liters and the acetone was replaced by hexane. The hexane solution was extracted with warm water and finally the solvent was removed by vacuum distillation. The yield was 1.7% of the original corn oil. The final product contained 52% unsaponifiable matter (completely precipitable by digitonin), 26% triglycerides, 21% free fatty acids and 1% moisture (volatile). Of this final product the daily ration per man was 1.7 g, containing 0.88 g of unsaponifiable matter, dissolved in imitation corn oil. The amount of unsaponifiable fraction given corresponds to that in 55 g/day of original corn oil.

Experimental design. Before beginning the experimental diets, in each experiment the men were maintained for about 6 weeks on a standard "House Diet" (see ref. 10, Table I). At the end of this control period, the matched groups were set up and they began subsistence on the experimental diets following the switchback design, one group starting with one diet and changing later to the second diet, while the other group subsisted on the diets in reverse order. In Exp. N the men were divided into 4 groups. Group W received safflower oil while Group X received corn oil; at the end of 3 weeks, the diets were reversed for another 3 weeks. Groups Y and Z were simi-

TABLE II. Serum Total Cholesterol Values, Means and Stand. Errors, on Corn Oil and 4 Other Vegetable Oils. Observed differences between corn oil and each of the other oils and the corresponding differences predicted from Equation 1, Δ Chol. $= 2.74\ \Delta S - 1.31\ \Delta P$.

Comparison				Cholesterol values, mg/100 ml				
						Δ corn *minus* other oil		Discrepancy, predicted *minus* observed
Corn oil *minus*	Exp.	No. of men	Period, wk	Corn oil	Other oil	Observed	Predicted	
Olive oil	J	14	2	160.7 ± 9.2	187.9 ± 8.4	−27.2 ± 3.6	−17.3	+ 9.9
Cottonseed oil	H	13	4	160.2 ± 9.0	179.5 ± 10.1	−19.3 ± 4.2	−11.3	+ 8.0
Sunflower seed oil	J	14	2	160.7 ± 9.2	170.0 ± 9.0	− 9.3 ± 2.8	+ 2.5	+11.8
Safflower oil	N	25	3	162.4 ± 5.5	159.7 ± 5.1	+ 2.7 ± 2.2	+ 9.1	+ 6.4

larly treated except that at the end of the first dietary period 2 other dietary periods were interposed before making the final switchback. In Exp. O after the control period the men were divided into 2 matched groups, A and B, both receiving the same diet for 6 weeks but during the first 3 weeks the men in Group A each received, daily, 1.7 g of unsaponifiable concentrate while Group B had this treatment in the second 3 week period. In all experiments blood was taken on 2 days near the end of each dietary period and serum analyzed for cholesterol(11).

Results. The results from switchback comparisons of olive *vs.* corn oil, cottonseed *vs.* corn oil, sunflower seed *vs.* corn oil, and of safflower *vs.* corn oil (Exp. N) are summarized in Table II. Note that in each experiment the diet was constant except for alternation of 2 types of oil fed at the level of 100 g daily/man, representing an average of 28% of total calories.

The most interesting feature of Table II is in the last column, "Discrepancy" between cholesterol differences observed with each pair of oils and the "predicted" difference. Obviously the prediction equation consistently predicts a higher cholesterol value on the corn oil than is actually found. Yet, as shown earlier (10), the prediction equation does not show such a consistent discrepancy with various amounts and kinds of fat (other than corn oil). The other fats and oils seem to form a homogeneous system in which corn oil does not fit precisely. Moreover, the magnitude of the discrepancy with corn oil is substantially the same in all experiments, 6.4 to 11.8 mg/100 ml, and the best estimate is that something in corn oil but absent from the other fats, results in average serum cholesterol about 9 mg% lower than expected when 100 g of corn oil are ingested daily. Alternatively, the other fats tested might contain something absent from corn oil that raises serum cholesterol, this "something" being substantially the same in effect in the other oils.

The results with the unsaponifiable fraction of corn oil are given in Table III. Every man showed a considerable fall in serum cholesterol in changing from house diet to either imi-

TABLE III. Serum Total Cholesterol, mg/100 ml Serum, at Ends of Dietary Periods in Exp. O. Each value is the mean of 4 measurements (duplicates on blood samples drawn on 2 different days).

House diet, control	Imitation corn oil	Imitation corn oil + unsaponifiable
208	165	160
238	184	185
178	156	155
169	147	144
221	164	150
148	126	118
204	179	170
160	135	140
239	202	203
234	196	197
179	147	153
252	200	187
254	196	182
258	194	174
222	170	192
148	132	127
262	198	179
192	165	180
178	131	151
168	140	151
234	171	165
199	154	158
178	140	125
172	138	138
229	174	154
250	206	189
206.7 ± 36.3*	165.8 ± 25.6	162.6 ± 23.2

* ± S.D.

tation corn oil alone or that oil plus the unsaponifiable fraction, the grand average change being –42.5 mg cholesterol/100 ml. Feeding the unsaponifiable fraction was associated with a trivial difference, 3.2 mg%, as compared with the oil mixture alone ($P = 0.17$). It is interesting, however, that the direction of this small difference is the same as in the other comparisons.

Discussion. These experiments demonstrate that the unsaponifiable fraction in corn oil cannot possibly account for the entire decrease in serum cholesterol, when corn oil is substituted for the mixed fats in an American diet. On the other hand, all these experiments consistently point to a small cholesterol-depressing effect of corn oil not accounted for by fatty acid composition, presumably produced by the unsaponifiable fraction, and not in olive, cottonseed, sunflower and safflower oils.

The average serum cholesterol discrepancy resulting from providing 100 g of corn oil in the daily diet is about 9 mg per 100 ml (Table II). From Exp. O the unsaponifiable material corresponding to 55 g of corn oil daily produced a discrepancy of 3.2 mg, suggesting that a discrepancy of 5.8 mg would be expected from the daily ingestion of the unsaponifiable material from 100 g of corn oil. These discrepancies are small, they are not statistically different from each other but they are all in the same direction. We conclude that the unsaponifiable fraction in corn oil probably accounts for a small part of the effect of corn oil on serum cholesterol. Considering both the experiment with isolated unsaponifiable matter and the discrepancies between observed and predicted cholesterol changes when corn oil was interchanged with four other vegetable oils, it is possible to estimate the special serum cholesterol lowering effect of 100 g of corn oil independent of fatty acids and primarily related to unsaponifiable compounds to be between 6 and 12 mg per 100 ml.

The concentrate of unsaponifiable matter obtained from the corn oil contained only about 50% of the total unsaponifiable present in the original oil and there is no way to tell whether the part obtained was more or less active than the part which was lost. We have assumed that the isolated unsaponifiable matter was a representative sample of the total.

These results are not inconsistent with two other series of experiments which have led to opposing conclusions. Beveridge *et al.*(2,8, 13) concluded that most of the effect was due to phytosterols (in the unsaponifiable fraction) in the corn oil, but Ahrens *et al.*(14) removed as much as 80% of the unsaponifiable material from corn oil and failed to demonstrate an alteration in the effect of the oil on the serum cholesterol. Data have not yet been published to support the conclusions of Beveridge *et al.* and the evidence from Ahrens *et al.* actually does not rule out the possibility of an effect of the magnitude found here.

Farquhar and Sokolow(15) have shown that the effects of plant sterols and of poly-ene fatty acids in lowering serum cholesterol are additive and this is also indicated here. The present experiments indicate that if practically all of the unsaponifiable material in corn oil consists of phytosterols, something like 3 g of such phytosterols ingested per day would produce a fall of the serum cholesterol of the order of 10 mg per 100 ml. This is, in fact, about the least dosage of sitosterol which is reported to result in a discernible change in the serum cholesterol(16).

In a recent trial corn oil tended to produce more cholesterol lowering than an equal amount of safflower oil(4). This finding, too, is consistent with the present results.

Summary. 1) Comparison of corn oil with 4 other oils of the linoleic-oleic acid type consistently indicated that corn oil in the diet produced slightly lower serum cholesterol values than those predicted, on the basis of fatty acid composition, by the multiple regression equation previously described. The mean discrepancy in 4 sets of switchback dietary comparisons with 13 to 25 men each and using 100 g of each oil in the daily diet was 9 mg/ 100 ml. 2) The source of this discrepancy was sought by feeding in a switchback experiment a daily supplement containing 0.88 g of unsaponifiable matter from corn oil, equivalent to that in 55 g of corn oil, added to a diet containing 140 g of fat of which 100 g was a mixture of safflower and cottonseed oils imitating corn oil. 3) Addition of unsaponifiable

matter produced a mean decrease of 3.2 mg of cholesterol/100 ml of serum. Although this effect is statistically non-significant, it matches the discrepancy observed in the earlier comparisons. It is concluded that the unsaponifiable matter of 100 g of corn oil lowers serum cholesterol by something between 6 and 12 mg/100 ml.

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Failure to Demonstrate Toxic Factors in the Serum of Irradiated Rats.*

(24070)

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Although circulating toxic humoral factors may play a role in radiation-induced illness, little unequivocal evidence supports such a factor(1). It has been demonstrated that serum from irradiated rabbits, incorporated into the media for growing chick heart fibroblasts and *Escherichia coli* depressed their rate of cell division(2). Van Dyke and Huff(3) reported that, in rats parabiotically united before irradiation of one of the parabionts with 900 r of X-radiation, epilation of both parabionts occurred. Non-irradiated parabiotic rats and single rats given 900 r did not show this effect. Lavik *et al.*(4), assumed that toxic factors were dialyzable but was not able to prolong, by *in vivo* dialysis, the lives of dogs which were given 500 r of whole body X-radiation. Whole blood taken at various time intervals post-irradiation from rats given supralethal amounts of whole body Co^{60} gamma radiation failed to cause weight changes or mortality in rats injected intravenously with this material(5). Recently Edelmann presented evidence indicating existence of lethal toxic substances in blood of adrenalectomized rats.‡ He reported that adrenalectomized rats and mice, injected intraperitoneally and intravenously with serum, plasma, and whole blood from other adrenalectomized irradiated rats (1000 r) died sooner than adrenalectomized animals which received blood from non-irradiated adrenalectomized donors.

The present experiments were initially designed to investigate physiological and histological effects of serum from irradiated animals. Before attempting to do this, serum from irradiated adrenalectomized rats was assayed along lines similar to those used by Edelmann. This report is concerned with results of this and other assay procedures. Since a toxic effect was not detected, histologic studies were not performed.

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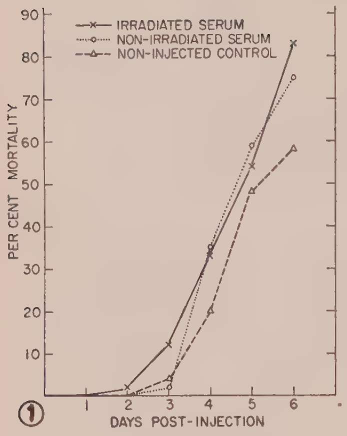
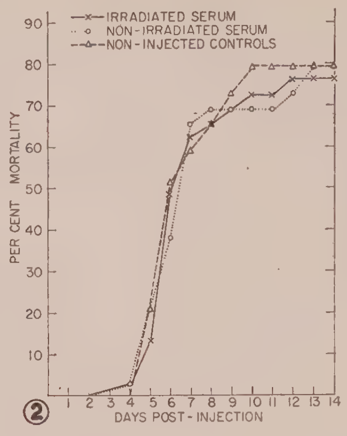
† Some data in this report were included in thesis submitted to Graduate School of St. John's University, N. Y., in partial fulfillment for degree of Master of Science. Present address: Predoctoral Fellow, Rockefeller Institute, N. Y.

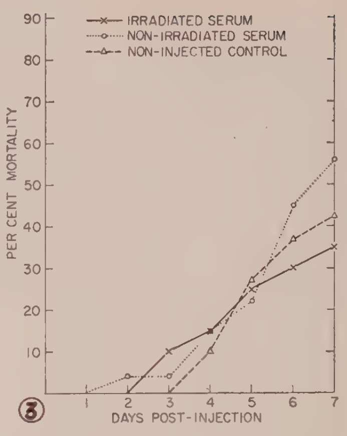
‡ Reported in *Fed. Proc.*, 1955, 42.

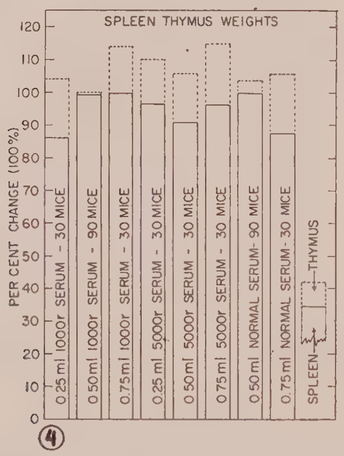
Materials and methods. Male and female Sprague-Dawley rat donors were adrenalectomized 1 or 2 days prior to irradiation and maintained on saline (1% NaCl) until irradiation and exsanguination. Sprague-Dawley rats and Carworth Farm recipient mice were adrenalectomized 1 or 2 days prior to injection. Adrenalectomy in all cases was performed through paravertebral lumbar incisions under sodium pentobarbital or ether anesthesia. Care was taken to remove the adrenal pedicle with a maximal amount of surrounding fat without damage to the capsule. Bleeding was absent or insignificant. The donors were irradiated with X- or gamma radiation 24 hours prior to exsanguination. Radiation factors were as follows: radiation was delivered by General Electric Maxitron therapy unit at 250 KVP; 30 ma; 0.5 mm Cu, 1 mm Al filter; HVl 1.5 mm Cu; TSD 70 cm. Tissue dose measurements were made with Victoreen chamber placed in box containing the animals; the tissue dose so measured was 70.4 r/minute. Gamma irradiation procedure has been described(6). Donors were given total body radiation of either 1000 r X-radiation or 1300 r gamma radiation. Exsanguination procedure was as follows: donor rats were anesthetized with ether until respiratory arrest; the abdominal cavity was opened and blood withdrawn from abdominal vena cava through a #20 hypodermic needle. A dry 10 cc syringe was used for each exsanguination. The blood was transferred to a dry glass centrifuge tube and allowed to clot for 30 minutes at 0-5°C. The clotted material was centrifuged at 1500 rpm at 2°C for approximately 30 minutes. The serum thus obtained was kept at 0-5°C until injection. All injections were made within one to 3 hours post-exsanguination. Intravenous injections were made via tail veins using #27 gauge needle. In all experiments involving adrenalectomized animals, pyrogen-free glassware and needles were employed for exsanguinations, blood processing, and injections. Pyrogen-free equipment was prepared in an oven at 170-180°C for 2 hours. Another type of assay involved degree of spleen-thymus weight loss, responsive to doses of radiation well below the lethal range(7). Three hundred and sixty intact female mice, 4 to 6 weeks old, were injected intravenously with serum from irradiated (1000 and 5000 r) and non-irradiated rats in doses of 0.25, 0.50, and 0.75 ml. There were 120 untreated control animals. Approximately 80 hours post-injection, the mice were sacrificed and spleens and thymi removed. Spleens were weighed on Roller-Smith torsion balance. Thymi were fixed in Carnoy's fixative, cleaned, and blotted semi-dry before weighing. In third type of assay, 650 r of Co^{60} gamma radiation was given to 90 mature, intact male CF-1 mice. Thirty of these received intravenously 0.6 ml of serum from adrenalectomized male and female rats which had been given 1300 r of Co^{60} gamma radiation 24 hours previously in attempt to increase mortality. One control group of 30 irradiated mice received the same amount of serum from non-irradiated adrenalectomized donors. Another control group of 30 was not injected. The mice were injected on seventh post-irradiation day. Observations for mortality were made daily for a 30-day period. Another group of recipient mice was given LD 22/30 dose (640 r) of gamma radiation and injected intravenously on 1st, 2nd, 3rd, and 4th post-irradiation day with 0.5 ml of serum from intact rat donors given 1000 r whole body X-radiation 1, 2, 3, and 4 days prior to bleeding.

Results. Fig. 1 represents results of 4 replicate experiments in which a total of 95 adrenalectomized rat recipients received intravenously 0.5 ml of serum from X-irradiated donors. Eighty-eight rats received serum from non-irradiated adrenalectomized donors and 69 others were not injected. Female donors and recipients were used; recipients weighed 40 to 80 g. There was no significant increase in mortality in group which received serum from irradiated donors. The apparent dif-









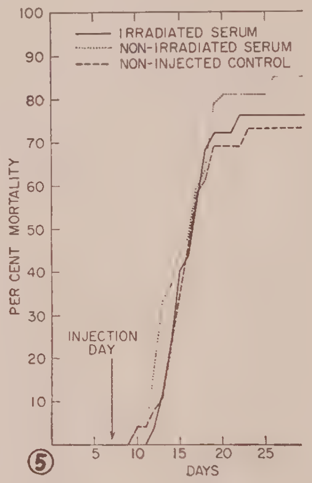


FIG. 1. Effect of intrav. administered serum (0.5 ml) from irradiated and non-irradiated adrenalectomized rats in adrenalectomized rat recipients.

FIG. 2. Effect of 4 ml of serum from adrenalectomized irradiated and non-irradiated rats administered intraper. to adrenalectomized rats.

FIG. 3. Effect of 0.6 ml of intrav. administered serum from irradiated and non-irradiated adrenalectomized rats in adrenalectomized mice.

FIG. 4. Spleen-thymus assay.

FIG. 5. Mortality effect of serum from adrenalectomized rats administered intrav. to pre-irradiated mice.

ference on third post-injection day is statistically insignificant.

Twenty-nine adrenalectomized female rats weighing 70 to 100 g and receiving intraperitoneally 4 ml of serum from other irradiated (1300 r gamma) adrenalectomized rats died at same rate as did 29 adrenalectomized rats which received 4 ml of serum from non-irradiated adrenalectomized donors and 29 non-injected adrenalectomized rats (Fig. 2).

There are no significant mortality differences in adrenalectomized mice which received 0.6 ml of serum from irradiated adrenalectomized rats and those which received the same amount of serum from non-irradiated donors or those which were not injected (Fig. 3). There were 30 animals in each group. Another experiment similar to this one, except that donors were adrenalectomized 48 hours prior to irradiation instead of 24 hours, gave same negative results.

The results of spleen-thymus assay are presented in Fig. 4. There was no significant atrophy of spleen or thymus after intravenous administration of serum from intact irradiated rats. Per cent change is measured from

100%, established on the basis of 120 non-injected control animals.

Fig. 5 shows that there was no potentiation of the effects of 650 r gamma radiation in mice by injection of serum taken from irradiated rats. This last type of experiment showed that there were no differences in mortality rate in pre-irradiated mice which received serum from irradiated rats during 24 hours to 96 hours post-irradiation. There was a minimum of 30 mice in each recipient group.

Discussion. The results of these experiments were negative. However, they signify little unless a reasonable estimate of sensitivity of the assay procedures can be determined, *i.e.*, are they sufficiently sensitive to give a qualitative indication of presence of radiotoxic material? To answer this question adequately, 2 assumptions must be made, (1) that toxic substances, if produced, would be carried in blood of irradiated animals and (2) that at some time after irradiation there would be sufficient toxic material in the circulating humor to produce observable effects in other animals. Since all blood and hence all toxic material will not be obtained and administered by ordinary exsanguination and injection procedures, a recipient test animal which is considerably more sensitive than normal should improve chances of detection.

It would seem likely that sensitizing an animal with lethal doses of radiation would make the animal more susceptible to deleterious effects of added radiotoxic material. Noble estimated that the threshold of response to any noxious agent by adrenalectomized animals is 5 to 20 times below that of their normal counterparts(8). Spleen and thymus involution resulting from very low doses of radiation would also appear to be a good biological criterion for measuring radiotoxicity of serum(7). These 3 sensitivity assays should be able to detect small amounts of toxic substances. Since no effect was detected, the hypothetical toxic substances were either in very low concentrations, slightly toxic or non-existent. At any rate these "radiotoxins" would appear of very little importance in the acute radiation syndromes. It is probable that large doses of radiation may result in liberation of physiologic or other substances capable of producing some transient and diverse physiological changes, particularly cardiovascular, following heavy exposure. It is clear from present experiments, however, that the hypoplasia and its sequelae seen with the syndrome of acute radiation injury is not mediated through liberation of a humoral toxic substance. The above considerations do not exhaust all possible alternatives. If toxic factors are extremely labile, they might be inactivated by handling. Associating sickness with toxic substances is not always apparently valid, *e. g.*, in avitaminosis. It is possible that an analogous situation exists in the irradiated animal. Toxic factors might be metabolized too rapidly for any detectable amounts to accumulate or they may diffuse out of the capillaries at a particular extravascular locus of action so that their presence in the blood would be of a very evanescent nature. Toxic factors might then be detectable at such locations or at a particular site of elaboration.

Summary. Three experimental procedures were utilized to assay serum from irradiated rats for radiotoxic substances. Such toxic material was not demonstrable by these technics. These experiments do not preclude the existence of radiotoxins in the irradiated animal, but they do indicate that if radiotoxins exist, the blood is probably not their principal storehouse. If, on the other hand, the blood does figure significantly in their storage, then they are not responsible for the severe signs and symptoms resulting from acute total body exposure.

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Effects of Altered Thyroid Function on Plasma Lipids of Experimentally Nephrotic Rats.* (24071)

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Occurrence of hypercholesteremia, increased tolerance to thyroid administration, and decreased metabolic rate in the nephrotic syndrome early implicated the thyroid gland in its pathogenesis(1). However, although the serum protein bound iodine is diminished in nephrotic patients(2), no actual abnormality of thyroid function appears to exist(3-5). The diminished protein bound iodine content of nephrotic serum appears to be due largely to its external renal loss(3-5), but also is believed by some to be partly due to deficiency of serum protein for its intravascular transport(3). It therefore appeared of interest to study the effects on nephrotic hyperlipemia of excess thyroid hormone administered in a *preformed* protein binding, and to determine the effects of preexisting thyroid dysfunction on plasma lipid changes occurring during subsequent induction of the experimental nephrotic state.

Methods. I. Effect of preexisting hyper- and hypothyroidism upon plasma lipids of rats injected with antikidney serum. A series of 32 male adult rats (Long-Evans strain) was made hyperthyroid by addition of 0.25% powdered thyroid substance to the stock ration for 18 days, and another series of 32 rats was made hypothyroid by addition of 0.3% powdered propylthiouracil to the diet for 6 weeks(6). The remaining 18 rats served as controls. Each rat was injected with 1 ml of pooled rabbit anti-rat kidney serum (AKS) (7). Groups of the hyperthyroid, hypothyroid, and control rats were sacrificed 24, 48 and 120 hours after injection of the AKS, at which time they were bled from the aorta for determination of plasma total lipids(8), total cholesterol(9), phospholipids(10), and albumin(11). Sections were obtained from the kidneys of the 16 rats sacrificed at the 48-hour interval after AKS injection and were stained with the Rinehart and Abul-Haj's modified Hale stain.

Results. Twenty-four hours after injection of AKS, the control rats exhibited average plasma total lipids of 464 mg/100 ml, total cholesterol of 133 mg/100 ml, and phospholipids of 220 mg/100 ml. These values were considerably higher and lower, respectively, in the hyper- and hypothyroid rats, despite their comparable injection of AKS (Table I). Similar differences were observed among the various groups of rats sacrificed 48 and 120 hours after AKS injection. Average plasma albumin concentrations were comparably lowered by injection of AKS in hyperthyroid and control rats, but were relatively less diminished in hypothyroid groups of rats.

Histological examination of the kidneys of the rats sacrificed 48 hours after AKS injection revealed characteristic glomerular changes and precipitated protein in the tubules(7), the changes observed in the hyper- and hypothyroid rats being of essentially comparable nature and degree to those of the control AKS-injected rats.

II. Effect of thyroid administration on plasma cholesterol of rats with preexisting nephrotic hyperlipemia. A. The effect of thyroid administration in nephrotic rats was studied by inducing the hyperthyroid state in 2 different ways. In the first experiment this was accomplished by parenteral administration of L-triiodothyronine. A series of 20 rats was injected with 1.0 ml pooled antikidney serum (AKS) and 7 days later the nephrotic rats were bled for plasma total cholesterol. Twelve of the rats then were injected subcutaneously with 100 μg of L-triiodothyronine daily for 4 days and on the fifth day were again bled for plasma total cholesterol. The remaining 8 rats served as controls. B. In the second experiment 10 additional rats were made nephrotic by injection of 1.2 ml of AKS and were bled for

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* Aided by grants from the Nat. Inst. of Health, P.H.S., and American Heart Assn.

TABLE I. Effect of Pre-Existing Hyper- and Hypothyroidism on Plasma Lipids of Rats Injected with Antikidney Serum.

			Avg plasma concentrations			
Type of rat	No. of rats	Avg wt (g)	Total lipids (mg/100 ml)	Total cholesterol (mg/100 ml)	Phospholipids (mg/100 ml)	Albumin (g/100 ml)
			Rats bled 24 hr after AKS inj.			
Hyperthyroid	17	180	741 ± 53.4* (400–1270)†	186 ± 9.0 (137–226)	397 ± 51.8 (253–546)	.7 ± .09 (.4–1.0)
Hypothyroid	15	230	267 ± 18.1 (161–402)	94 ± 4.7 (67–112)	151 ± 13.6 (138–237)	1.6 ± .19 (.8–2.4)
Control	13	236	464 ± 48.2 (230–790)	133 ± 8.1 (98–196)	220 ± 37.2 (191–264)	.7 ± .10 (.5–.9)
			Rats bled 48 hr after AKS inj.			
Hyperthyroid	5	190	1261 ± 112.2 (1109–1534)	237 ± 19.7 (203–284)	459 ± 64.0 (378–615)	.8 ± .25 (.7–.9)
Hypothyroid	6	224	504 ± 86.2 (208–712)	135 ± 21.4 (71–231)	181 ± 25.2 (118–250)	1.5 ± .23 (1.0–2.0)
Control	5	212	1413 ± 254.1 (466–2035)	170 ± 21.5 (100–240)	255 ± 29.3 (167–350)	.9 ± .19 (.7–1.1)
			Rats bled 5 days after AKS inj.			
Hyperthyroid	10	191	2112 ± 297.0 (586–3291)	330 ± 53.3 (183–464)	646 ± 95.2 (446–850)	.9 ± .05 (.8–1.0)
Hypothyroid	11	225	644 ± 65.3 (200–987)	211 ± 21.5 (110–333)	295 ± 17.6 (224–356)	1.6 ± .04 (1.5–1.8)

* Stand. error of mean. † Range of values.

plasma total cholesterol one week later. During the ensuing 8 days, 0.4% powdered thyroid substance was added to the ration of 5 of the rats, the remaining rats serving as controls. At the end of the experimental interval the rats were again bled for plasma total cholesterol.

Results. A. Injection of L-triiodothyronine did not appear significantly to affect plasma cholesterols of the rats with preexisting nephrotic hypercholesteremia. Thus although the average plasma total cholesterol of the treated rats fell during the experimental interval from an initial average of 323 mg/100 ml (Range: 222-502) to a final average of 158 mg/100 ml (Range: 75-219), a comparable decrease was observed in the control rats whose plasma cholesterol similarly fell during the experimental interval from an initial average of 371 mg/100 ml (Range: 170-493) to 189 mg/100 ml (Range: 91-278).

B. Addition of a relatively large amount of thyroid substance to the ration of the second series of rats with preexisting nephrotic hypercholesteremia possibly exerted a greater effect than did L-triiodothyronine. Thus the plasma total cholesterol of the treated rats fell from an initial average of 628 mg/100 ml (Range: 451-816) to 168 mg/100 ml (Range: 167-176), representing an average decrease of 460 mg/100 ml (Range: 281-640; S.E. Mean ± 84.6). At the same time average plasma cholesterol of the control rats fell from 414 mg/100 ml (Range: 256-577) to 274 mg/100 ml (Range: 31-439; S.E. Mean ± 67.6). However, although the difference in the 2 groups was statistically significant, cholesterol changes in both groups of rats varied so considerably that observed results are questionable.

III. Effect of repeated administration of serum obtained from hyperthyroid rats on plasma lipids of nephrotic rats. The inconclusive effect of induced hyperthyroidism in the latter experiments may have been partly ascribable to absence in the nephrotic rat's plasma of adequate protein for intravascular hormone transport(3). To determine the possible effect of administering to nephrotic rats an excess of thyroid hormone in a *preformed* protein binding was of interest.

Methods. Hyperthyroidism was induced in a group of rats by adding 0.4% powdered thyroid substance to their diet for 21 days. The

TABLE II. Effect of Repeated Injection of Hyperthyroid Rats' Serum on Plasma Lipids of Nephrotic Rats.

			Avg plasma concentrations				
			Initial		Final		
Type of inj. rat serum	No. of rats	Avg wt (g)	Total lipids (mg/100 ml)	Total cholesterol (mg/100 ml)	Total lipids (mg/100 ml)	Total cholesterol (mg/100 ml)	Albumin (g/100 ml)
Rats with moderate nephrotic hyperlipemia							
Hyperthyroid	5	176	465 (175–790)*	142 (117–196)	531 (405–1000)	96 (68–170)	
Control	5	184	445 (230–710)	122 (111–137)	517 (250–810)	104 (60–161)	
Rats with severe nephrotic hyperlipemia							
Hyperthyroid	5	182		314 (234–435)	882 (584–1260)	281 (219–369)	.8 (.5–1.0)
Control	5	190		316 (176–420)	1001 (892–1150)	262 (205–366)	.7 (.4–.9)

* Range of values.

rats then were bled and their pooled serum found to contain a protein bound iodine content of 16.8 μg/100 ml in contrast to a concentration of 4.0 μg/100 ml in a second pool of rat serum obtained from untreated normal rats. A series of 10 rats then was injected with 1.0 ml of AKS. One week later these nephrotic rats were bled for plasma total cholesterol and total lipids. Five of the nephrotic rats then were injected daily intravenously with 3 ml of the pooled donor hyperthyroid rat serum, the remaining 5 control rats being similarly injected, but with the pooled serum obtained from the normal rats. Six days later the rats were bled for determination of plasma total lipids and total cholesterol. A similar experiment was performed in a second series of 10 rats in which a more severe hyperlipemic state was induced by injection of 0.75 ml AKS on each of 2 consecutive days. One week later, 5 of the rats were injected daily with 3.0 ml of the pooled hyperthyroid rat serum and the remaining 5 control rats with the pooled normal rat serum, as in the first series of rats. Plasma total cholesterol was determined prior to the first serum injection and plasma cholesterol, total lipids and albumin after 6 daily injections of the donor serum.

Results. The results are presented in Table II. The slight changes observed in both series of nephrotic rats injected with the pooled serum obtained from the donor hyperthyroid rats were closely similar to those occurring in control rats injected with normal rat serum.

Discussion. The acute administration of excess thyroid hormone, despite its route, did not appear to alter the elevated plasma lipids of nephrotic rats under the present experimental circumstances. A similar lack of apparent response to prolonged thyroid therapy has been noted in nephrotic human subjects (12). This failure of thyroid administration to lower the elevated lipid content of nephrotic plasma might be ascribed in part to renal loss of administered hormone(3-5) and to the fact that, despite the occurrence in such plasma of subnormal content of protein bound iodine (2), normal thyroid function is present in the nephrotic syndrome(3-5). Conversely, administration of excess thyroid hormone is known to be capable of lowering the plasma lipid content of normal human subjects(13), as well as that of normal rats(6). Therefore, the present failure of thyroid exhibition to lower the elevated lipid content of the nephrotic rat's plasma also suggests that there is a metabolic block in the plasma egress of the various lipid fractions, which cannot be overcome by administration of excess thyroid hormone.

Our previous studies of the mechanism of nephrotic hyperlipemia have shown that such a metabolic block in the plasma egress of lipid does indeed occur in the nephrotic state(14, 15). These studies have indicated that the critical deficiency of circulating albumin in nephrotic plasma is primarily responsible for failure of normal lipolysis and plasma clearance of triglyceride, which results in intravascular accumulation of excess triglyceride(16-20), and associated secondary plasma retention and accumulation of excess phospholipid, cholesterol, and cholic acid characteristic of such plasma(19-20).

On the other hand, administration of excess thyroid hormone to *normal* rats was shown in previous studies from this laboratory to so markedly increase their rate of plasma elimination of cholesterol that, despite the concomitant markedly increased rate of cholesterol synthesis in such hyperthyroid rats, their resultant plasma content of cholesterol is lowered(6). This would also appear to be true of the plasma phospholipid content in the hyperthyroid state(21,22). Therefore, if a metabolic block in the intravascular egress of cholesterol and phospholipid were to be introduced during an existing state of hyperthyroidism, it might be anticipated that their markedly increased rates of synthesis consequent to the hyperthyroid state(6,21) would lead to progressive hypercholesteremia and hyperphospholipemia. This sequence was indeed found to occur in the present studies when such a metabolic block to lipid egress from the plasma was introduced by injection of antikidney serum(19). Thus it was observed that the prior presence of an established hyperthyroid state markedly augmented the rate of rise of plasma cholesterol and phospholipid otherwise occurring during early development of the nephrotic syndrome, a response which could not be ascribed to any apparent effect of the hyperthyroid state on renal changes or degree of hypoalbuminemia effected by injection of the antikidney serum in these rats.

A considerably slower rise of plasma lipids of lesser magnitude occurred in hypothyroid rats injected with antikidney serum, despite occurrence in such rats of glomerular changes of comparable nature and degree to those observed in the control and hyperthyroid rats similarly injected with antikidney serum. Although this altered response might be partly ascribable to the slowed rate of lipid synthesis in the hypothyroid state(6), it is probably mainly a consequence of the fact that less severe depletion of the plasma albumin content occurred in these rats. The results agree with earlier findings that, although elevated plasma lipid levels are inversely related to lowered plasma albumin content of nephrotic plasma (18,19), plasma lipid-albumin concentrations in such plasma are not a linear function, significant hyperlipemia occurring only in the presence of *severe* depletion of the circulating albumin content.

Summary. 1) The effect of a preexisting state of hyperthyroidism and of hypothyroidism on plasma lipid changes occurring during development of experimental nephrotic syndrome was studied in rats. Conversely, the effect of excess thyroid administration on plasma lipids of rats with previously established nephrotic hyperlipemia was studied. 2) Under present experimental circumstances, acute administration of excess thyroid hormone, orally, parenterally or intravenously in a preformed protein binding, did not significantly affect the elevated plasma lipids of nephrotic rats. On the other hand, the rise of plasma lipids occurring during development of nephrotic state in rats was markedly augmented in presence of preexisting hyperthyroidism, and conversely, was considerably slowed by prior induction of an hypothyroid state. The mechanism of these responses was briefly discussed.

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Bicarbonate Reabsorption along Renal Tubules.* (24072)

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Montgomery and Pierce first demonstrated that in the frog kidney urine is acidified in the distal tubule(1). More recently it has been suggested that the mechanism of acidification is one involving an exchange of hydrogen for sodium. Such a system is also invoked in reabsorption of bicarbonate from tubular urine. Pitts(2) and Berliner(3) suggest that hydrogen exchanges for sodium along the entire nephron, *i.e.*, in proximal as well as distal tubules. Nicholson(4) demonstrated by histochemistry that the brush border of proximal tubules may be quite acidic, suggesting that hydrogen ions are secreted into urine by proximal cells as well as distal. Using the stop flow technic devised by Malvin, Wilde and Sullivan(5,6,7), we have located along the nephron the transport systems for bicarbonate.

Principle of stop flow analysis. Administration of an osmotic diuretic mannitol to a dog establishes such a high rate of urine flow that concentrations are changed to a new steady value. If during this period the ureter is clamped shut, intratubular pressure rises until filtration at the glomeruli ceases. During this interval of stopped flow urinary concentrations will be altered as static columns of tubular urine remain trapped in the various nephron segments. If glucosuria is developed before occlusion, such that glucose reabsorptive transfer maximum is exceeded, the occlusion will allow further time for lowering glucose concentrations along the proximal tubule. If para-aminohippurate (PAH) is included in the infusion, the occlusion will allow further time for increasing the proximal PAH concentrations. In the distal areas occlusion will allow the Na concentration to be lowered. On release of occlusion the diuretic flush washes the concentration pattern along each nephron. Serial urine samples segment this pattern into an ordered array, best pictured on a graph if concentration of each sample is plotted against accumulated volume of urine which has appeared before the particular sample since reinstatement of flow. Plots against time show a distortion due to variable flow rates. The first samples to enter the collector come from distal areas and show decreasing concentrations of Na. As proximal fluid begins to enter, the Na concentrations rise, while glucose

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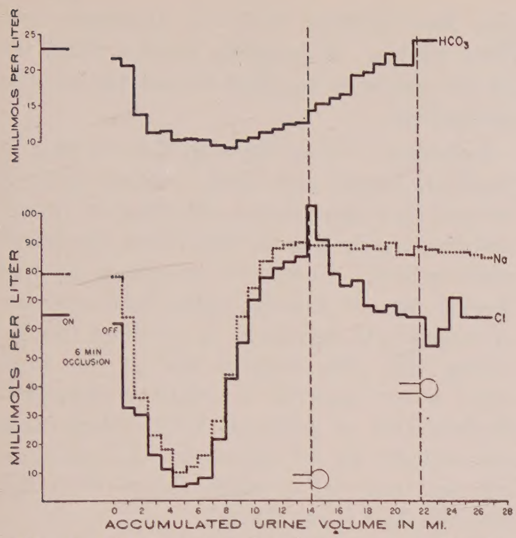

FIG. 1. Urinary stop flow patterns for bicarbonate, chloride and sodium.

concentrations fall. The lowered glucose concentration pattern overlaps that of rising PAH. Wilde and Malvin(7) have developed a graphical method of deriving the locus of transport segments along the nephron from these concentration patterns.

Methods. Six experiments were done on mongrel dogs weighing 12-16 kg, anesthetized with 35 mg/kg of sodium pentobarbital injected intraperitoneally. One ureter was exteriorized through flank incision and catheterized well into the pelvis with polyethylene tubing. A constant intravenous infusion was supplied at 10 ml/min. Infusions contained 15-20% mannitol plus PAH, creatinine and sufficient sodium bicarbonate, so that this anion appeared in all urine samples. All infusions were made up to volume with Ringer's solution. When urine flow stabilized at some high level the experiment was begun. Bicarbonate concentrations were determined using a Kopp-Natelson microgasometer. Chloride was determined using the method of Brun(8), creatinine by Bonsnes and Taussky(9), PAH according to Smith(10). Sodium was determined using Beckman direct reading flame photometer.

Results. Fig. 1 depicts a bicarbonate pattern typical of all 6 experiments. Urinary concentration along the ordinate is plotted against accumulated volume of urine which appeared after reinstatement of flow and before the particular sample. To the left of the break in each curve are plotted control concentrations of various urinary solutes during free diuretic flow. Solute concentrations to the right of break represent concentrations elaborated along the tubule during stopped flow as released in the new urinary washout pattern. The 2 vertical dashed lines bearing filter diagrams encompass that part of the volume scale over which more and more new filtrate mixes with the proximal stop flow pattern. The left line indicates the position of the filter of the shortest nephron; the right line represents the level of the filter of the longest nephron. The position of these filters can be located by the rising concentration of inulin injected after ureteral occlusion and which thus lies at the glomeruli ready to mark entry of new filtrate(6,7). More conveniently they were marked with PAH, the enormous proximal secretion of which forms a concentration peak at the first filter (14 ml on Fig. 1). A sample at this 14 ml level is most typical of true stop flow proximal fluid(7), being minimally contaminated with distal fluid from the longer nephrons or with new filtrate soon to enter the shortest nephrons.

Sodium (Fig. 1) reached its lowest concentration at 5 ml, representing delivery of samples from a maximum number of low sodium distal nephron segments. At the 14 ml landmark, sodium has already returned to the concentration plateau typical of free osmotic diuresis, indicating a uniquely weak proximal reabsorption(6,7) of sodium away from the water bulk held by mannitol.

Bicarbonate, as is sodium, is strongly reabsorbed at the distal 5 ml level but unlike sodium does not rise to the free flow plateau concentrations in the best proximal sample (14 ml). Its concentration is low here (14 ml) and then rises slowly as more and more new filtrate mixes with less and less of the low stop flow proximal concentration. It rises to free flow values at 22 ml just as the last of low-bicarbonate, stop flow proximal fluid from the longest nephrons is reaching the urine collector. This behavior proves that stop flow proximal concentrations of bicarbonate are lower than new flow or free flow proximal values(7).

While chloride also is strongly reabsorbed at the 5 ml distal site, contrary to bicarbonate, it is relatively elevated in the proximal area. In 2 experiments Cl lies parallel to Na in the proximal area. In the 4 others, Cl concentrations rise to a peak at level 14 ml just as does PAH (Fig. 1). This is the criterion for secretion, or that stop flow chloride concentrations in the proximal are higher than new flow concentrations in this area(7).

In their micropuncture studies in rats and guinea pigs, Walker, Bott, Oliver and MacDowell(11) found proximal chloride concentrations elevated over those in plasma. They presumed that HCO_3 was preferentially reabsorbed.

Additional proximal reabsorption of $NaHCO_3$ by itself during stop flow should carry out water osmotically leaving the remaining chloride elevated over free flow urinary concentration by the same amount as the bicarbonate drop. Actually the chloride rise exceeds this. Part of the chloride rise may likewise result from reabsorption of sodium salts of phosphate, sulfate, etc., with water following to isotonicity.

In some experiments the mass of Cl associated with non-transportable creatinine in the proximal stop flow samples, exceeds that remaining with creatinine in the free flow samples, *i.e.*, our ratio $(U_{Cl}/U_{cr})/(P_{Cl}/P_{cr})$ is higher in proximal samples during stop flow (See definition in Ref. 6) than for urine of free flow. It seems possible that HCl is secreted into the proximal tubule during stop flow thus titrating $NaHCO_3$, phosphate and other buffers. A complete balance sheet for all ion species is required to test the various alternatives.

Summary. Using the stop flow technic in $NaHCO_3$ loaded dogs under osmotic diuresis we find that bicarbonate and chloride are reabsorbed avidly in a far distal area coincident with sodium. Compared to values in free flowing osmotic diuretic urine, concentration of bicarbonate developed in proximal tubules during stop flow remains low at the same point where chloride is relatively elevated. Reabsorption of proximal HCO_3 alone does not account for Cl elevation. Cl secretion into and removal of other anions from the proximal fluid is considered.

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